UNIVERSITY OF CALIFORNIA SAN DIEGO

PTPN2 Inhibitors Sensitize Melanoma Tumor Cells to Immunotherapy

A Thesis submitted in partial satisfaction of the requirements for the degree Master of Science

in

Biology

by

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The Thesis of Rachel Tang is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Co-chair

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ABSTRACT OF THE THESIS

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by

Rachel Tang

Master of Science in Biology

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Professor Amy Pasquinelli, Co-chair

In recent clinical studies, the success of immune checkpoint inhibitors to modulate the immune system in metastatic melanoma patients has shown a great response rate. However, there are still subsets of patients which are only partially responsive or non-responsive towards this cutting-edge immunotherapy. This unpredictable therapeutic response has caused a set-back to treating this deadly disease. A phosphatase, Protein Tyrosine Phosphatase Non-Receptor Type 2 (PTPN2) has been implicated in reducing tumor cells responses to immunotherapy; deletion of PTPN2 in tumor cells positively influenced the efficacy of immunotherapy by increasing interferon-γ-mediated effects on antigen presentation and growth suppression. By employing a virtual high throughput screening (vHTS) and computer-aided structure-drug design, PTPN2 inhibitors were designed and synthesized. Ten small molecule

inhibitors of PTPN2 were analyzed in murine melanoma models and three inhibitors, ID_5, ID_7, and ID_9, were found to have potent activity against PTPN2. Ptpn2 inhibition by these lead compounds induced the intracellular Interferon-γ (IFN-γ) signaling pathway. Furthermore, these inhibitors enhanced Stat1 phosphorylation, defining the underlying mechanism of inhibitors in Interferon signaling. Additional cell viability assays, quantitative reverse transcription PCR (RT-qPCR) analysis, and western blotting of rationally designed Ptpn2 inhibitors demonstrated their *in vitro* anti-melanoma cancer activities with no apparent toxicity in murine cell lines. While further investigations are needed using *in vivo* models, these data altogether indicate that Ptpn2 small molecule inhibitors are novel agents that may enhance therapeutic responses in melanoma cancer which are resistant to currently available immunotherapies.

Chapter 1: Introduction

1.1 Cancer

Cancer is still the leading cause of death worldwide, where new cases arise instantaneously [4]. With the proliferation of medical treatments for cancer, the overall cancer death rate in the United States fell by 25% from 1990 to 2014 [3]. According to the National Cancer Institute, the number of cancer survivors in the United States is predicted to increase from 15.5 million in 2016 to 20.3 million by 2026 is extraordinary[3]. Personalized therapeutic treatments for each individual cancer patient has changed the way clinicians and researchers search for cures for this deadly disease. Despite the availability of a wide array of treatments, cancer is still able to find its way around many chemotherapies and increase the number of new cancer cases to 23.6 million by 2030 [3]. Overall, the decrease of cancer mortality rate due to a myriad clinical trials provided by advances in cancer research has allowed prolongation of patients' survival rate. Nevertheless, there is still much work needed in the fight against this disease, as the major cause of each case is still unknown and more questions are being generated along with the new knowledge gathered.

1.1.1 Hallmarks of Cancer

Unlike sickle cell disease which is due to a point mutation in the genome, cancer is caused by multiple mutations that happen sequentially or together. To this day, researchers are still unable to pin point a specific cause or causes of this disease. Indeed, cancer has proven to be highly variable at the molecular level, and therefore each patient has diverse responses to therapy. As depicted in Figure 1, there are a set of phenotypic properties for cancer to develop from uncontrolled cell proliferation, forming benign tumor to cells capable of metastasizing nearby or distant organs.

Hallmarks of cancers by Hanahan and Weinberg (2011), has shown that the occurrence of cancer is due to multiple reasons such as somatic mutations (insertion, deletion and point

mutation in the genome), chromosomal rearrangements and epigenetic alteration (DNA methylation and demethylation) which has increased the complexity of treating cancer. Its genetic heterogeneity due to various mutations in key regulatory genes such as tumor suppressor (P53) or ligand independent receptors have promoted tumorigenesis leading to cells resisting apoptosis, sustaining autocrine proliferative stimulation and metastasizing cancer. As normal cells ostensibly proceed to neoplasm state, the tumor microenvironment is also affected where immune cells (natural killer cells, T-cells and B-cells) are being disabled to allow cancer cells to evade destruction [1].

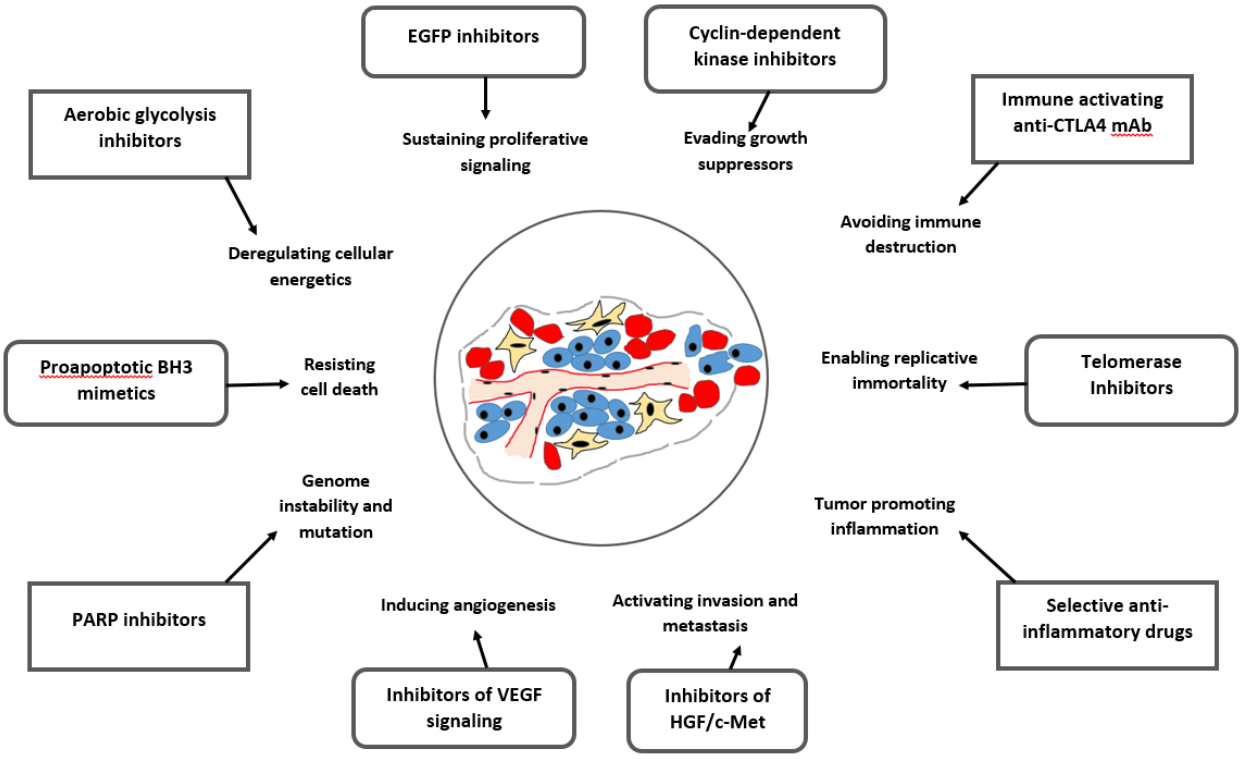


Figure 1.1: Hallmarks of Cancer [1].

1.2 Metastatic Melanoma Epidemiology

Skin cancer is among the most common cancer types according to statistics collected by the National Cancer Institute during 2018. Melanoma accounts for only about 1% of skin

cancers but causes a large majority of skin cancer deaths [5]. The rate of melanoma cancer has been rising rapidly for the past 30 years. It is estimated that in the United States for 2018, about 91,270 new melanomas will be diagnosed and 9,320 people are expected to die due to melanoma [5].

The human skin is composed of multilayered cells and tissues coming together to provide protection and regulation against harmful factors. It predominantly consists of three main layers: the epidermis, dermis and subcutaneous layer where fatty tissues mainly reside. The melanocyte cells are where melanin pigments are produced which gives skin its tan color. Without melanin, ultraviolet ray from the sun will cause harmful damage to cells below the epidermis which leads to melanoma cancer.

External (ultraviolet ray and viruses) and internal factors (hormones or hereditary mutations) may sequentially come together to promote carcinogenesis due to genetic mutations in melanocyte cells. One of the major risk factor leading to malignant neoplasm of the melanocytes is due to blistering sunburn [6]. Frequent ultraviolet radiation exposure causes DNA mutation in melanocytes such as the formation of pyrimidine dimers or deamination of cytosine into thymidine, eventually leading to melanoma tumorigenesis [6].

1.2.1 Subtypes of Melanoma

With the tremendous studies being performed on the histology and pathophysiology of the various subtypes melanoma, the classification of each melanoma according to clinical and morphological features of tumors has provided insight to the disease. The three common histogenic subtypes of cutaneous melanoma. Superficial spreading melanoma (SSM) is the most common type of cancer where 50-70% of cases are in younger patients [8]. These malignant melanocytes have a lateral metastasis characteristic throughout the epidermis. This is followed by the nodular melanoma (NM) cases which accounts for 15-30% of melanoma cases

and is found on a patient's neck and head [8]. Unlike SSM, this subtype of melanoma proliferates vertically similar to a protruding ulcer. Lastly, Lentigo maligna melanomas (LMM) occurs in about 5-15% of melanoma patients, where the skin has been damaged due to over exposure of the sun [8]. Histologically, the growth of multinucleated melanocytes is mainly lentiginous and atrophic epidermis [8].

Traditional therapies for the treatment of early or late stage melanoma generally involves surgical excision, radiation and chemotherapy. However, all these treatments have proven to be ineffective with low overall cancer patients' survival rate. In addition, chemotherapies approved by the Food and Drug Administration (FDA) for treating melanoma have cytotoxic effects towards patients which do more harm than good. Finally, after all the years of laborious studies into immunotherapy, there is now a light at the end of the dark tunnel called cancer.

1.3 Immunotherapy

Chemotherapy, has changed tremendously where nowadays individualized treatment is tailored for each patient specifically as compared with a generic treatment for all patients. Further understanding of the mutation landscapes of cancer has allowed advancement of targeted therapies for subtypes of tumors with significant outcomes. The field of immunotherapy goes all the way back to 1891 when Dr. William B. Coley was the first to develop immunotherapeutic strategies for humans. Dr. Coley inoculated bacteria into sarcoma patients which stimulated sustained activated antitumor immune response to suppress cancer proliferation [9]. This has laid the foundation for current discoveries of immunotherapies that have been included in patients' treatment regimens.

When a foreign antigen is found within a person's body, the immune system will engage by identifying and eradicating the foreign substance with a balance of necessary processes for a controlled immune response. Tumor cells release antigens that are captured by dendritic cells to present the tumor's antigen on major histocompatibility class 1 (MHC1) or major histocompatibility class 2 (MHC2) complexes. Antigen presenting cells (APC) are the dendritic

cells with MHC1 or MHC2 where they present the tumor's antigens for cytotoxic T-cell (CD8+) recognition [16, 17, 18]. Due to the cancer cells' aberrant genetic and cellular processes, the normal host's immune system will be able to differentiate between "self" and "nonself" antigen. Hence, the normal host CD8+ effector T-cells will eliminate cancer cells by amplifying T-cell responses [16, 17, 18]. Nevertheless, cancer cells are still able to escape and suppress the immune system recognition. These findings have raised questions regarding the approaches and strategies to inhibit and overcome cancer.

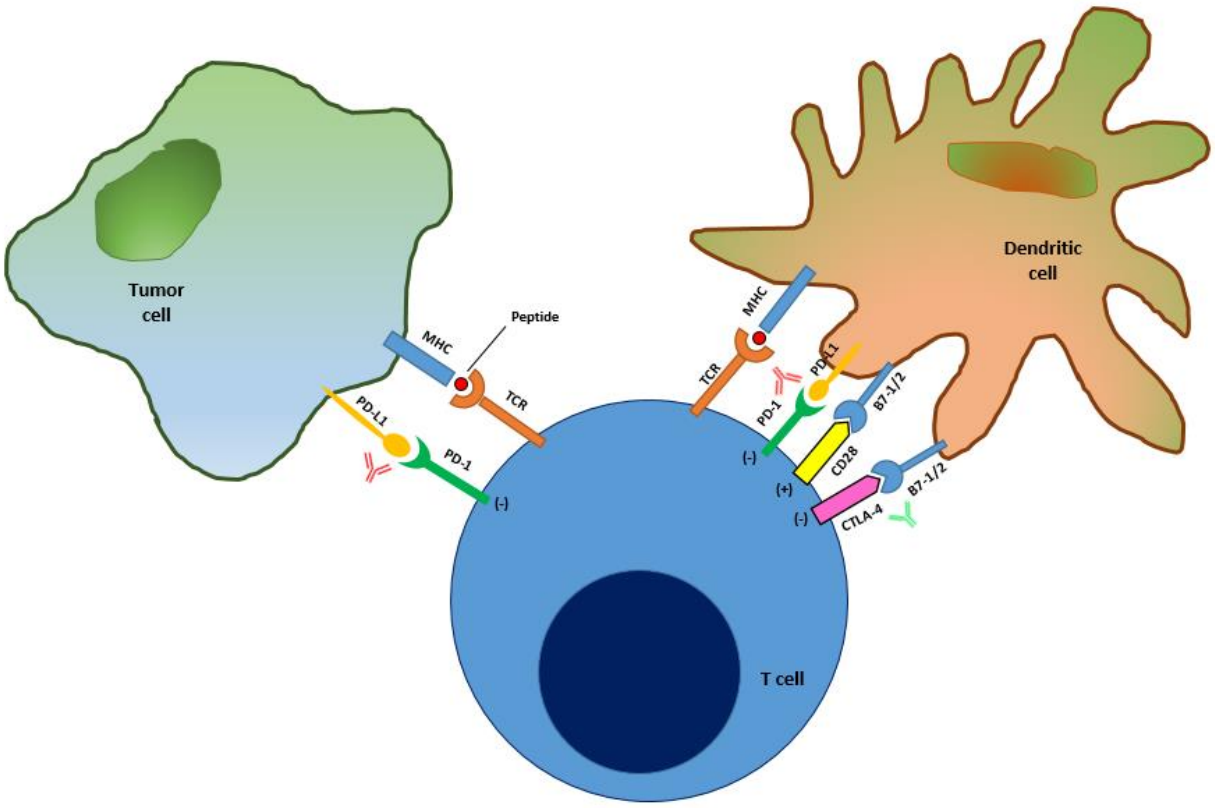


Figure 1.3: Phagocytosis of apoptotic cells by an antigen-presenting cell (APC) often are dendritic cell leading to the presentation of antigen on MHC. Interactions between CD28/B7 promote T-cell receptor (TCR) to recognize peptide on MHC (present on tumor cells) resulting in amplification and activation of cytotoxic T-cells. CTLA4/B7 and PD-1/PDL1 interactions between dendritic cells and T cells leads to T cell exhaustion. Hence, tumor cells escape elimination by host's immune system. With the presence of anti-PD-1/PD-L1 and anti-CTLA4 prevents cancer cells evading T-cell mediated apoptosis [18, 19].

In recent years, more specific immunotherapies have been approved by FDA, such as antagonistic monoclonal antibodies, chimeric antigen receptor T-cell (CAR-T) and oncolytic

virus. Out of the many immunotherapies that have been used since 2010 within the United States, immune checkpoint inhibitor PD-1/PD-L1 and CTLA4 pathways have been in the spotlight as they have response rates of 20-30% across myriad tumor types [15].

These promising results have led many to prioritize these therapies by combining with other immunotherapies such as cancer vaccines. Despite all these positive results, there are still pressing challenges where patients are not responding to treatments. Transforming non-responders to responders to immunotherapy remains a question which many laboratories are trying to solve.

1.4 PTPN2: A novel target to enhance immunotherapy

Despite all the new and exciting avenues of treatments being potential cures for cancer patients, checkpoint blockade does not provide sustainable response to many patients. Recently, the discovery of Ptpn2 gene as an inhibitor of immunotherapy response has been reported by Manguso et al. using pooled loss-of-function genetic screens in vivo [2]. Wide scale genetic screens using CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)-associated nuclease Cas9 to study functional consequences of gene deletion for a large number of genes controlling tumor cells proliferation [10].

PTPN2, Protein Tyrosine Phosphatase Non-Receptor Type 2 (also known as TCPTP) dephosphorylates proteins such as Janus-activated kinases (JAK)-1 and -3, Signal transducer and activators of transcription (Stats)-1, -3 and -5, and Epidermal growth factor receptor (EGFR). This leads to inactivation in a cell type according to context [21]. A classical catalytic domains of protein tyrosine phosphatases (PTPs) has approximately 280 residues comprising 22 invariant and 42 highly conserved residues [21]. With its highly conserved “SHP [SH2 (Src homology2)-domain-containing protein tyrosine phosphatase]-1 and -2 PTPs or TCPTP (T-cell PTP) and PTP1B” has “demonstrated substrate specificity where it is overexpressed in cancer cells promoting to tumorgenesis” [21]. Loss-of-function of Ptpn2 in human T cell acute

lymphoblastic leukemias and triple-negative breast cancer cause tumor proliferation rapidly through constitutive JAK and STAT signaling [20].

Much research has been carried out in many different cancer types where deletion of Ptpn2 leads to tumor growth and metastases [2]. Interestingly, this has been contradicted in a recent study performed by Manguso et al. [2]. According to Manguso et al., PTPN2 deletion is beneficial in promoting sensitization of tumor cells to immunotherapy [2]. When CD8+ T cell and natural killer (NK) cell release interferon-γ (IFN-γ) to eliminate tumor cells, cancer cells counter attack by increasing PD-L1 expression in response to cause T cell exhaustion. IFN-γ causes phosphorylation of Stat1 Y701 mediated by JAK1/2 which allows translocation of Stat1 to the nucleus and increases transcription of PD-L1 in tumor cells [2]. As a negative regulator of IFN-γ signaling, Ptpn2 suppresses tumor immunity by dephosphorylating and inactivating both phosphorylated JAK1(Y1022/Y1023) and Stat1(Y701) (Figure 1.4) [2, 21].

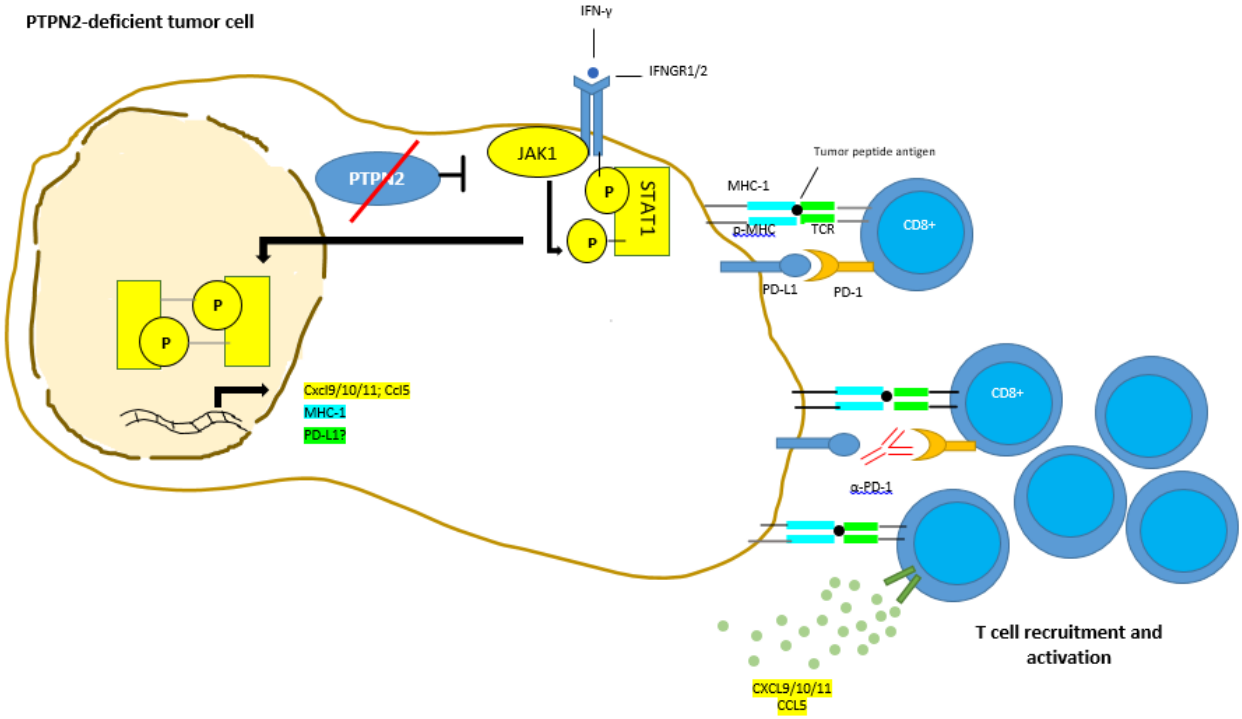


Figure 1.4: PTPN2 deletion in murine tumor cells promotes phosphorylation of Stat1 protein to translocate into nucleus. Null Ptpn2 tumor cells treated along with immune checkpoint blockade anti-PD-1 enhances susceptibility to immunotherapy [20].

In Manguso et al. research, Ptpn2-null cancer cells have demonstrated dramatic effect on the susceptibility of tumor cells to immunotherapy through anti-PD-1 [2]. This has simultaneously increase the recruitment of cytotoxic CD8+ T cells with the increase antigen presentation cells to activate more T cells. The deficiency of Ptpn2 in tumor cells has upregulated gene expression along the IFN-/STAT1 pathway, including T cell chemoattractants (CXCL9, CXCL10, CXCL11 and CCL5) and components of the antigen-processing and presentation pathway [2, 21].

Altogether, these findings have provided the rationale to designing Ptpn2 inhibitors as potential targets to enhance the outcome of immunotherapy. Studies in this thesis present the inhibitory activities, cell toxicity, and mechanism of PTPN2 inhibitors. With these rationally designed compounds, it may be possible to treat selective patients' tumors by overcoming their resistance to immunotherapy.

1.5 Aims of the study

The loss-of-function of protein tyrosine phosphatase Ptpn2 in murine tumors has shown great efficacy to PD-1 treatments where monoclonal antibody antagonistically block immune-inhibitory receptor on tumor cells and amplify immune response. Hence, producing small molecules inhibiting Ptpn2 would be ideal in the quest to prolong the patients' overall survival by enhancing immunotherapy responses.

- Create Ptpn2 knockout melanoma cells
- Evaluate cytotoxicity effects of compounds to melanoma cells
- To determine changed activities in enhancing IFN-γ responses
- To assess the mechanism by which the inhibitors exerts its effects against melanoma cells

Chapter 2: Results

2.1 Establishing stable knockout cell lines

The main purpose for establishing a stable knockout Ptpn2 cell line in B16 melanoma murine cell line is to establish a positive control. Ptpn2-null was generated using the Zhang lab's lentiCRISPv2 backbone vector where it has hSpCas9, the single guide RNA (sgRNA) and a puromycin selection marker into B16 cells (Figure 4.1). Loss-of-function mutation of Ptpn2 with RNA-guided CRISPR (Clustered Regularly Interspaced Short Palindrome Repeats) – along with nuclease Cas9 was very effective due to low off-target effects. This lentiviral vector was chosen instead of a cell line expressing Cas9 as it is faster and easier since one vector has the ability to express both Cas9 and sgRNA simultaneously through single transduction [10].

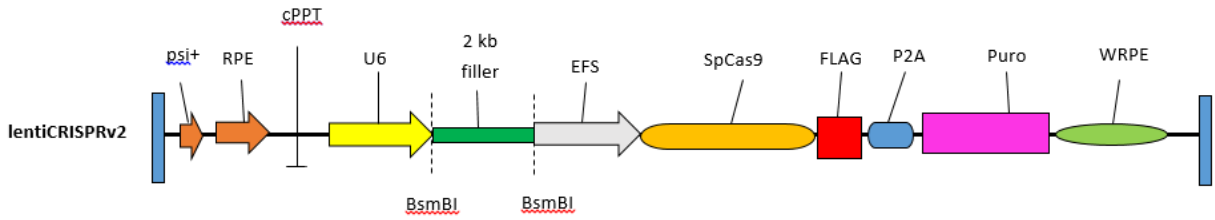
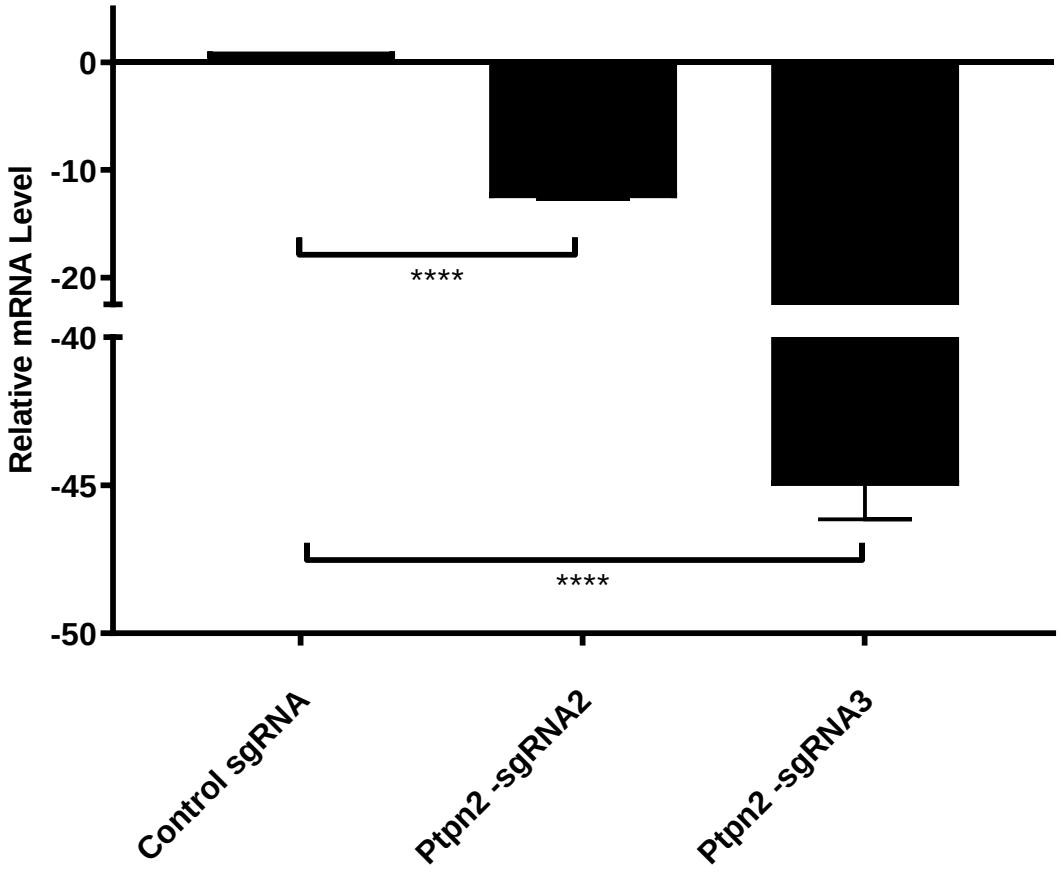


Figure 2.1: Lentiviral vector with which has Cas9 protein and sgRNA sequence annealed into the 2kb filler. Psi+, psi packaging signal; RPE, rev response element; cPPT, central polypurine tract; U6, U6 promoter; EFS, elongation factor-1α short promoter; P2A, 2A self-cleaving peptide; Puro, puromycin selection marker; WPRE, posttranscriptional regulatory element [10].

Target sequences targeting Ptpn2 were cloned into the lentiCRISPRv2 backbone by synthesizing two oligonucleotides with BsmBI enzyme's overhangs. Oligonucleotides were reconstituted with DNase and RNase free sterile water to achieve 100μM. Following the Zhang lab's protocol [10], backbone vectors were digested and dephosphorylated with BsmBI enzyme first. Annealing of target sequence into vector was performed before transformation into Stbl3 bacteria [10]. Plasmids collected went through gene sequencing by Genewiz to make sure oligonucleotides were ligated correctly into the backbone vector.

Lentivector with packaging plasmids were transfected into HEK293(F)T cells through transient transfection to produce lentivirus for transduction into the target cell line. Each transduced cell line went through puromycin selection. In order to determine the efficacy of gene knockout through lentiCRISPR, four Non-Targeting Control (NTC) sgRNA and two Ptpn2 sgRNA stable cell lines were quantitated with real-time qPCR (RT-qPCR) (Graph 2.1).

Graph 2.1: Relative mRNA level of Ptpn2-null relative to NTC B16 cells by qPCR. Data are mean ± s.e.m., n=4 for each data point, one-way ANOVA, ****P<0.0001.



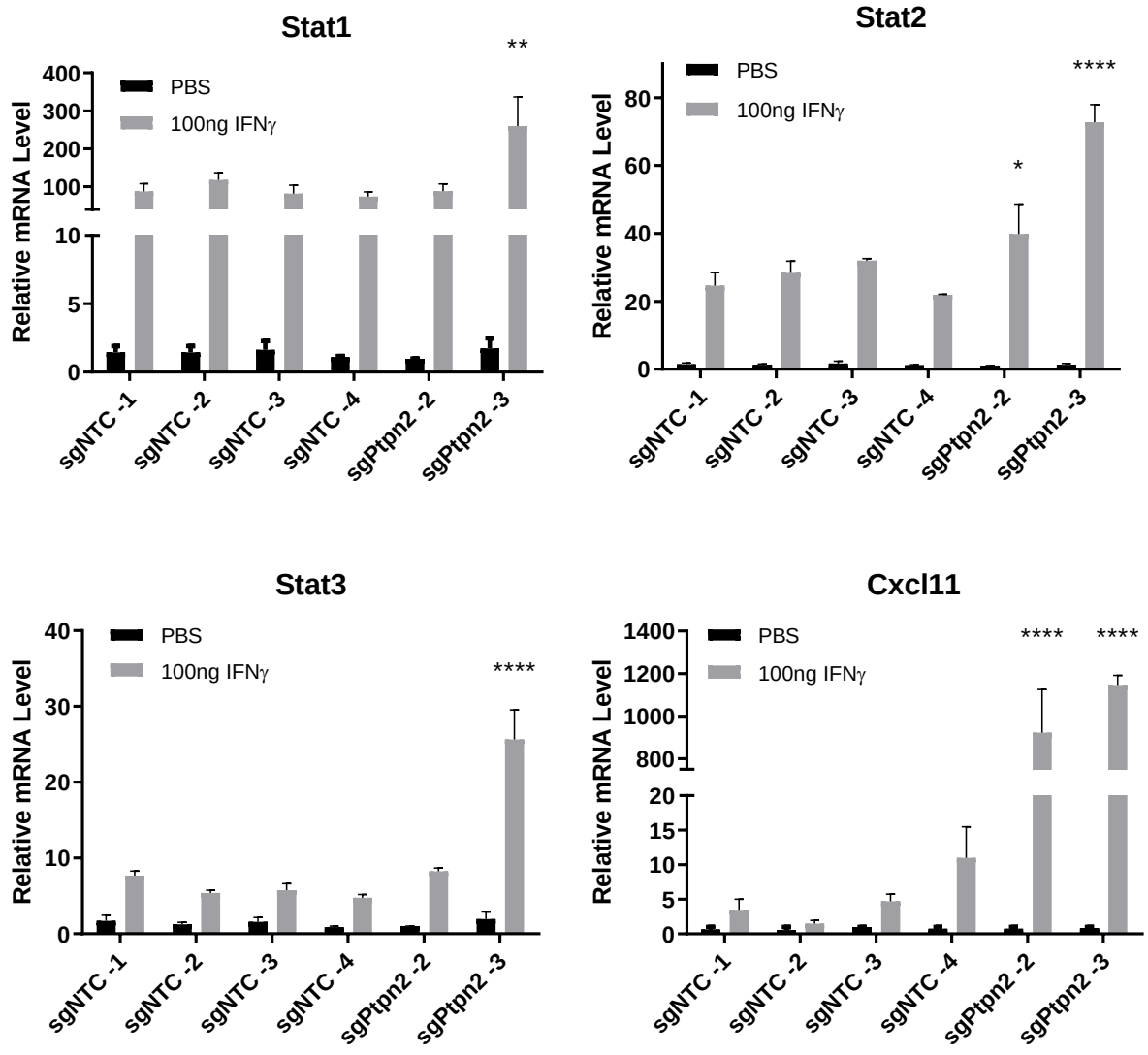
2.2 Loss of Ptpn2 sensitizes tumor cells to cytokines treatment

According to Manguso's *et al* [2], Ptpn2 is a protein tyrosine phosphatase where it negatively regulates intracellular process in IFN-γ pathway through inhibition of dephosphorylation of JAK1 and STAT1 [2]. Hence, the loss-of-function of Ptpn2 sensitizes tumor cells to immunotherapy by increasing IFN- γ signaling [2].

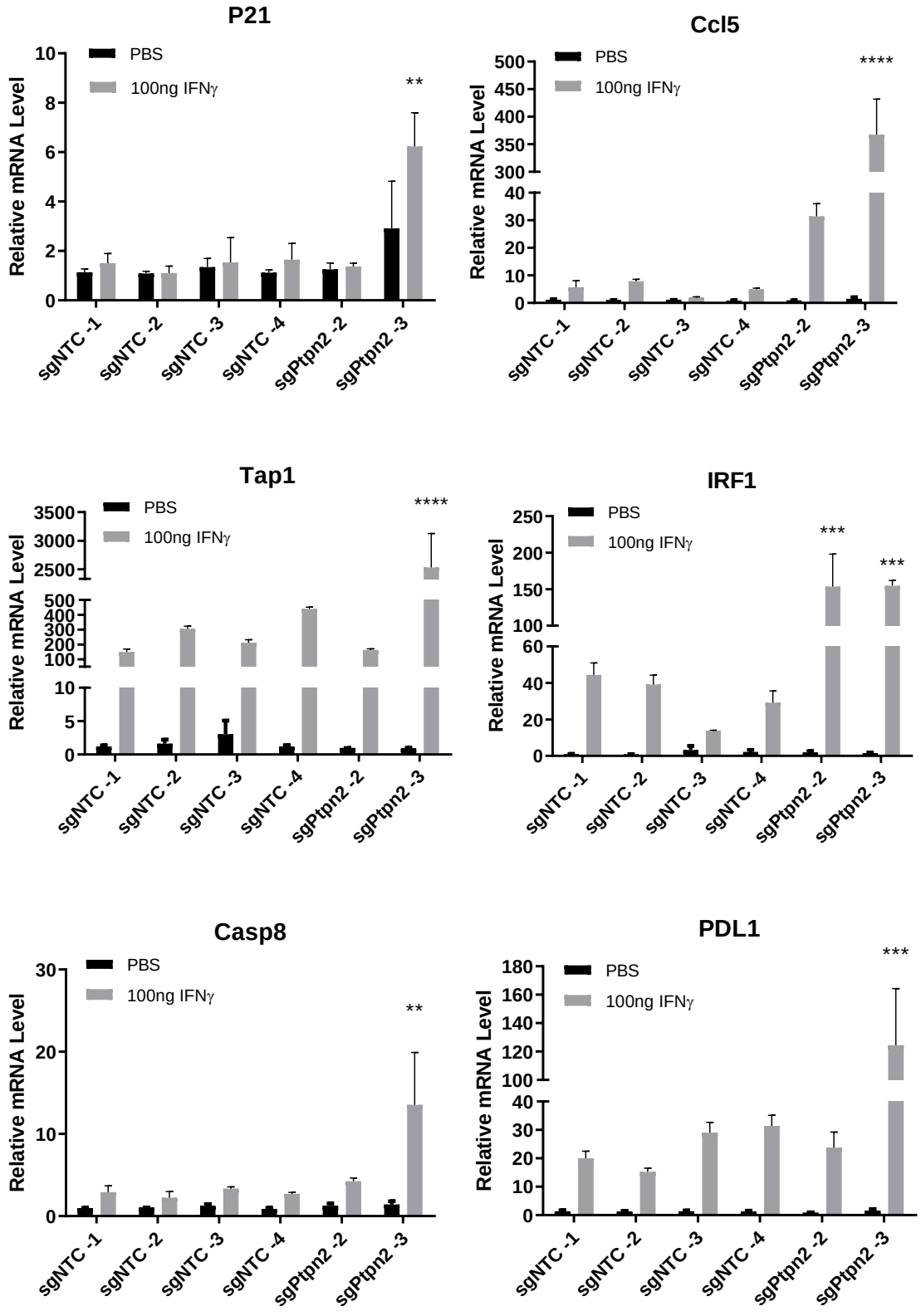
Knockout Ptpn2 stable cell lines were treated with interferon-γ and tumor necrosis factor-α (TNF-α) to optimize conditions such as number of cells in each wells and concentration of cytokines. The knockout stable cell lines were treated to assay Stat1 activity during cytokines stimulation. Ptpn2 loss of function along with NTC-cell line were seeded into 12-well plates where it achieves about 50% confluency the following day. The cells were treated with 100ng/ml IFN-γ cytokine dissolved in cell culture media for 72 hours of incubation. Prior to 72 hours of incubation, the media in each well was replaced with fresh media containing cytokine.

The transcriptional RNA levels in knockout Ptpn2 and control B16 cells after being exposed to 100ng/ml of IFN-γ, is shown in the Graph 2.2. Of the many genes that were upregulated in Ptpn2-null cells, several T cell chemokines (Cxcl11 and Ccl5; Graph 2.2) are potentially involved in the increasing T cell infiltration into tumor. Downstream pathway gene expression such as Stat1, Stat2, Stat3 and IRF1 of IFN-γ were also upregulated. Moreover, Tap1 and PD-L1 were also upregulated in Ptpn2-null cells which are members of antigen presenting pathway. Increase in cellular response to cell-cycle regulators such as Casp8 were also quantified in Ptpn2-null tumor cells treated with cytokines relative to NTC-melanoma cells. Moreover, both have shown an increase in Stat1 phosphorylation after treatment with IFN-γ.

Graph 2.2: IFN- γ downstream pathway gene expression in sgNTC (control) and sgPtpn2 (Ptpn2 –null) cell lines. All assays were performed in duplicate, and all the experiments were repeated at least two times (mean ± s.e.m., n=4 for each data point, two-way ANOVA, *P< 0.0332, **P<0.0021, ***P<0.0002, ****P<0.0001 in comparison with NTCs).



Graph 2.2: Continued



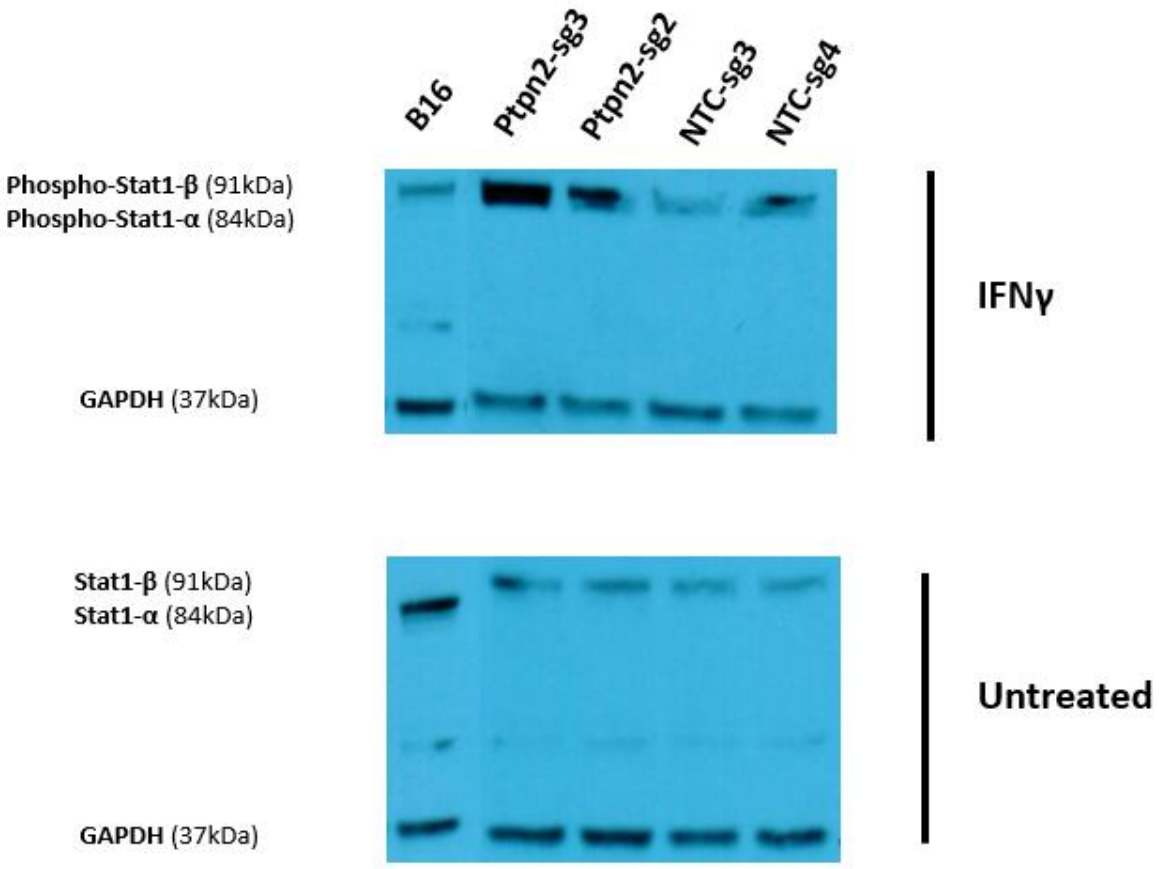
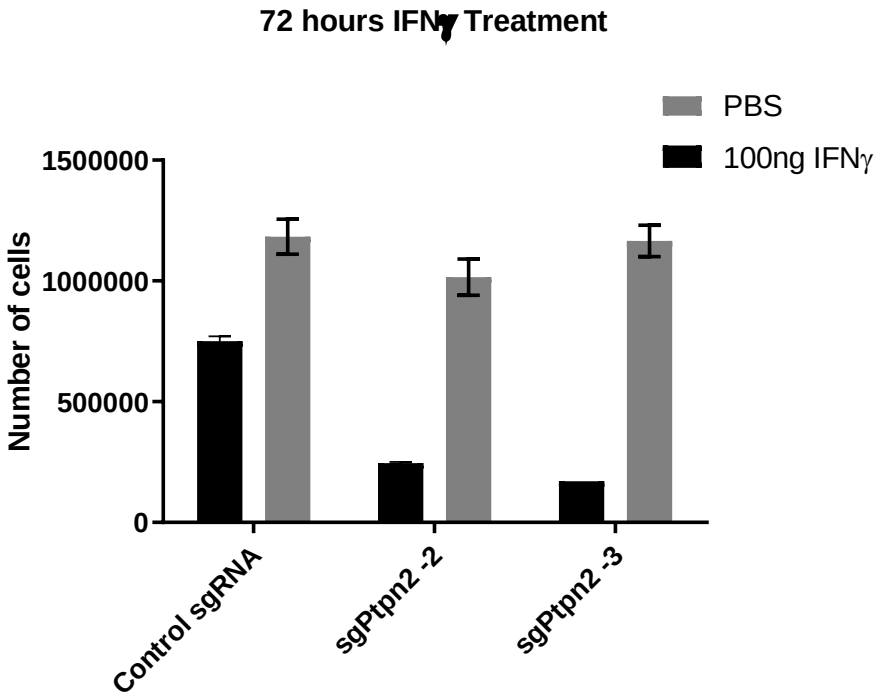


Figure 2.2: Ptpn2 deletion enhances phosphorylation of Stat1.
After 24 hours treatment with 100ng/ml of IFN-γ, an increase of phosphorylated-Stat1 in Ptpn2-sgRNA2 and sgRNA3 was observed in comparison with the control.

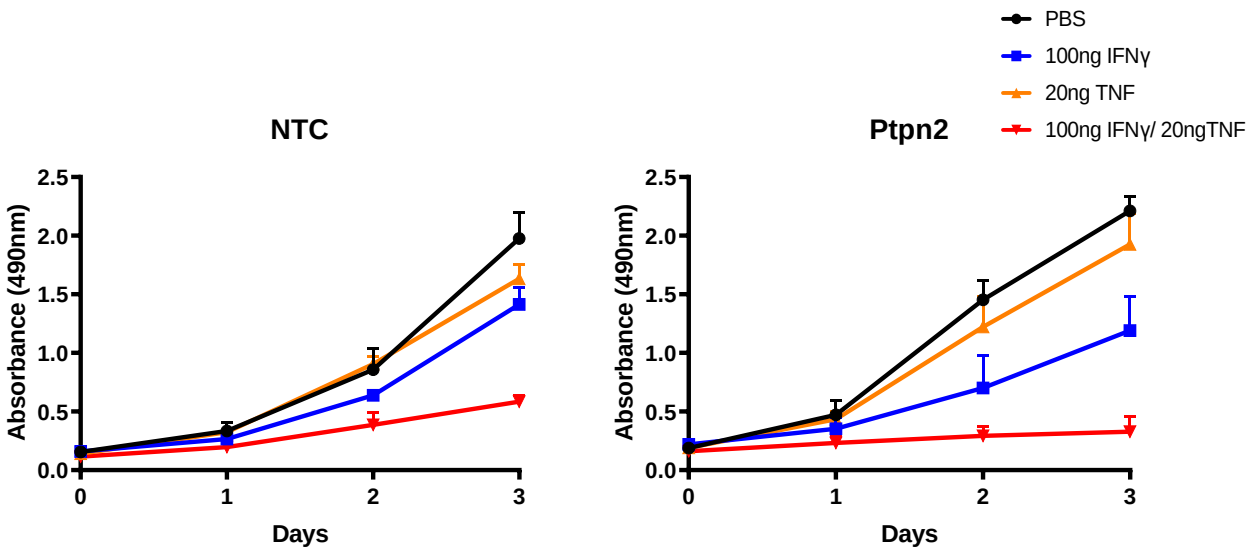
In addition, cell counting through trypan blue and MTT assays were performed on Ptpn2-null cells treated with cytokines to study cell proliferation and viability. In comparison to proliferation assay which uses trypan blue dye to detect blue apoptotic cells' compromised membrane and counting viable cells, the MTT assay reduces MTS tetrazolium compound to purple formazan through production of NADPH and NADH by dehydrogenase enzyme of metabolic living cells. A day before treatment, 50,000 cells were seeded and the number of cells was calculated after 72 hours incubation with IFN- γ. As shown in Graph 2.3, the number of live cells present had decreased significantly in Ptpn2-null cells unlike the control and untreated cells. Additionally, MTS assays were performed on Ptpn2-null cell lines treated with culture

medium containing cytokines for three days: (i) 100ng/ml IFN-γ; (ii) 20ng/ml TNF-α and (iii) combined 100ng/ml IFN-γ with 20ng/ml TNF-α. A reduction in cell growth and viability were observed in IFN-γ and combined treated Ptpn2 null murine cell lines (Graph 2.4). Both Graphs 2.2.2 and 2.2.3 have shown that untreated Ptpn2-null cells were proliferating at a similar rate to untreated control B16 cells. Conversely, both assays have also shown a decreased in the growth of knockout Ptpn2 unlike NTC-B16 cells when treated with IFN-γ.

Graph 2.2.2: Proliferation rates of control and Ptpn2-null cells measured after IFN-γ treatment through trypan blue cell calculation with hemacytometer.



Graph 2.2.3: Cell viability of control and Ptpn2-null cells were performed using MTS assay in triplicates. Cell viability was measured every 24 hours for three days.



The figures below showed the knockout Ptpn2 and control B16 cells being treated in all the three different settings: (i) 100ng/ml IFN-γ; (ii) 20ng/ml TNF-α and (iii) combined 100ng/ml IFN-γ with 20ng/ml TNF-α. The cells in 96-well images were taken at 24 hours, intervals for three days. After the first 24 hours treatment with IFN- γ, the cells had very slow growth. Unlike the cells treated with TNF-α, which proliferated very similar to the cells treated with only PBS, the IFN- γ/ TNF-α combined treated cells have many cell death. These results demonstrates that IFN- γ stimulation alone can sensitize and cause disadvantage growth to melanoma cells lacking Ptpn2. With this optimized condition for treating cells of interest with cytokines, Ptpn2 inhibitor assay was performed next.

(A)

Control and Ptpn2-null stable cell line with cytokines treatment (0 Days)

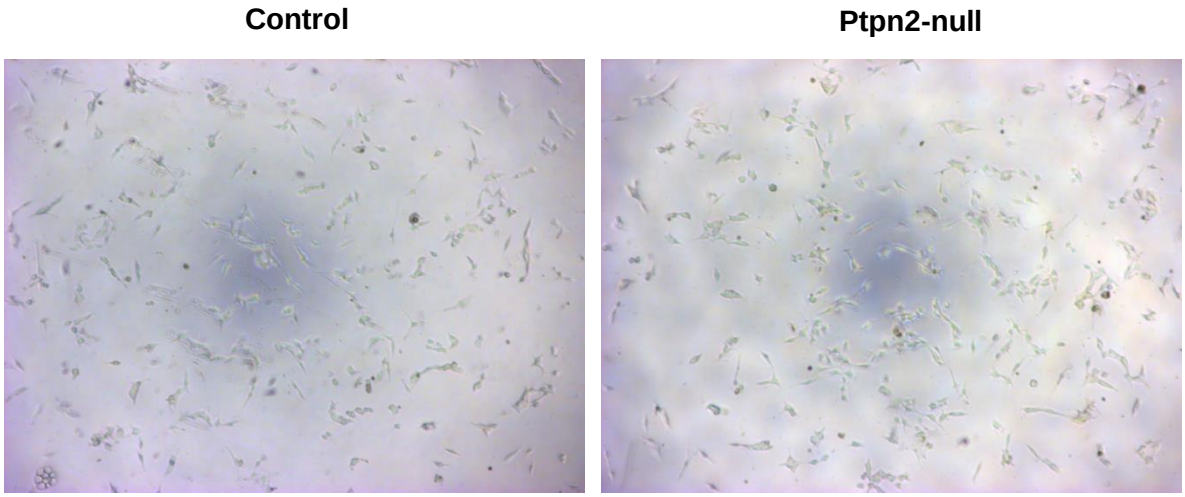


Figure 2.2.2: Images of control and Ptpn2-null tumor cells after treated with cytokines: **(A)** Day 0, **(B)** Day 1, **(C)** Day 2, and **(D)** Day 3.

(B)

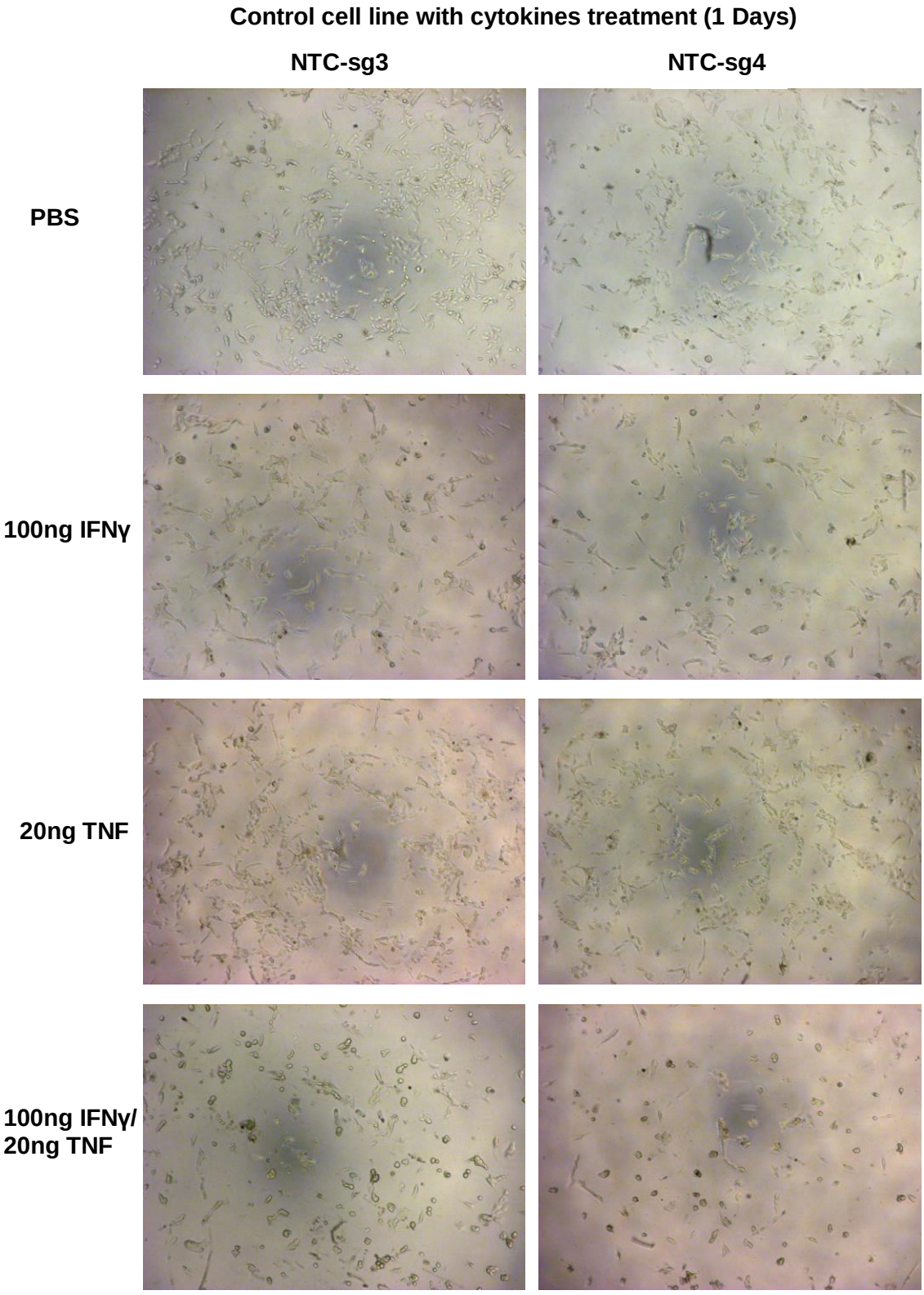


Figure 2.2.2: Continued

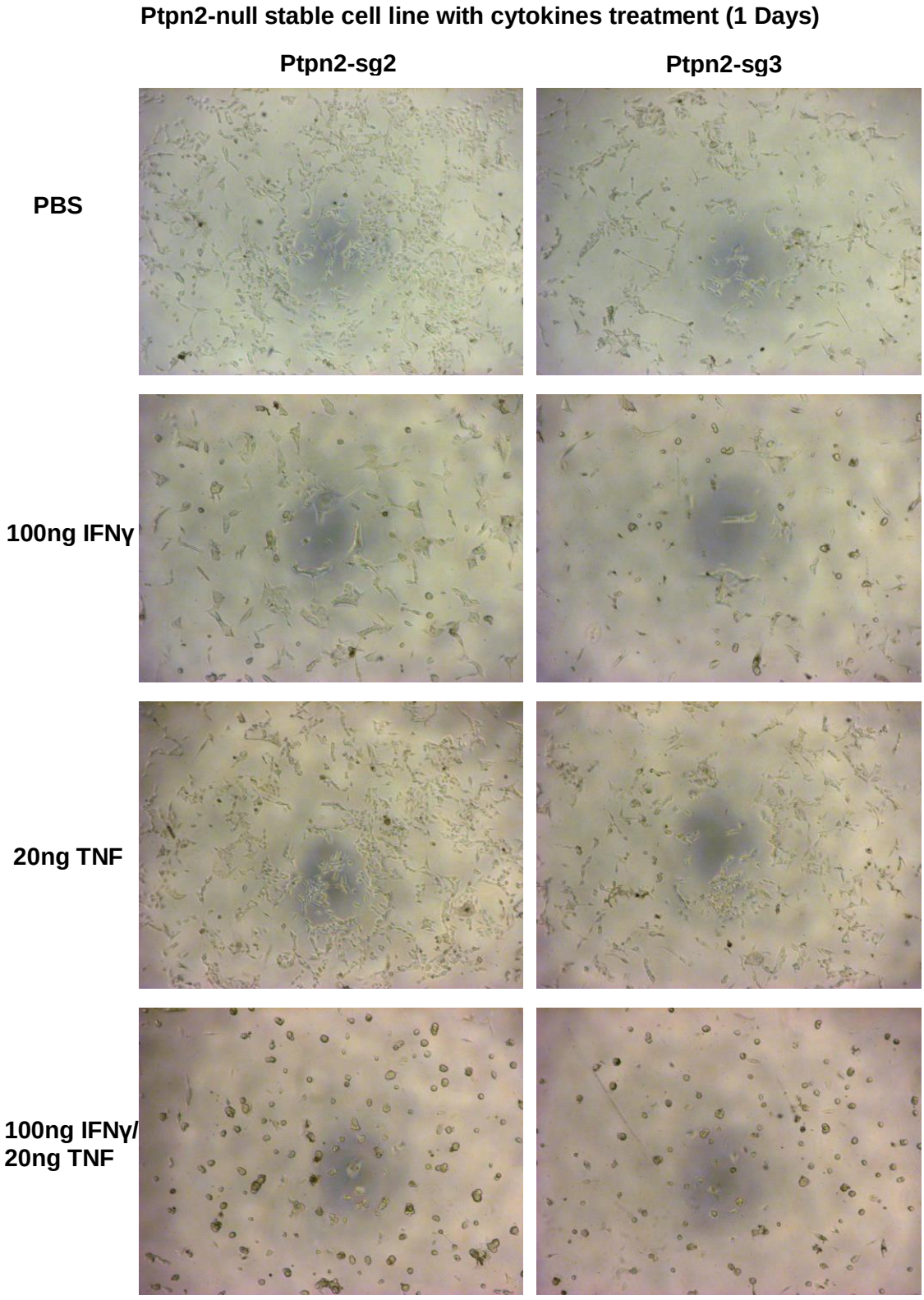
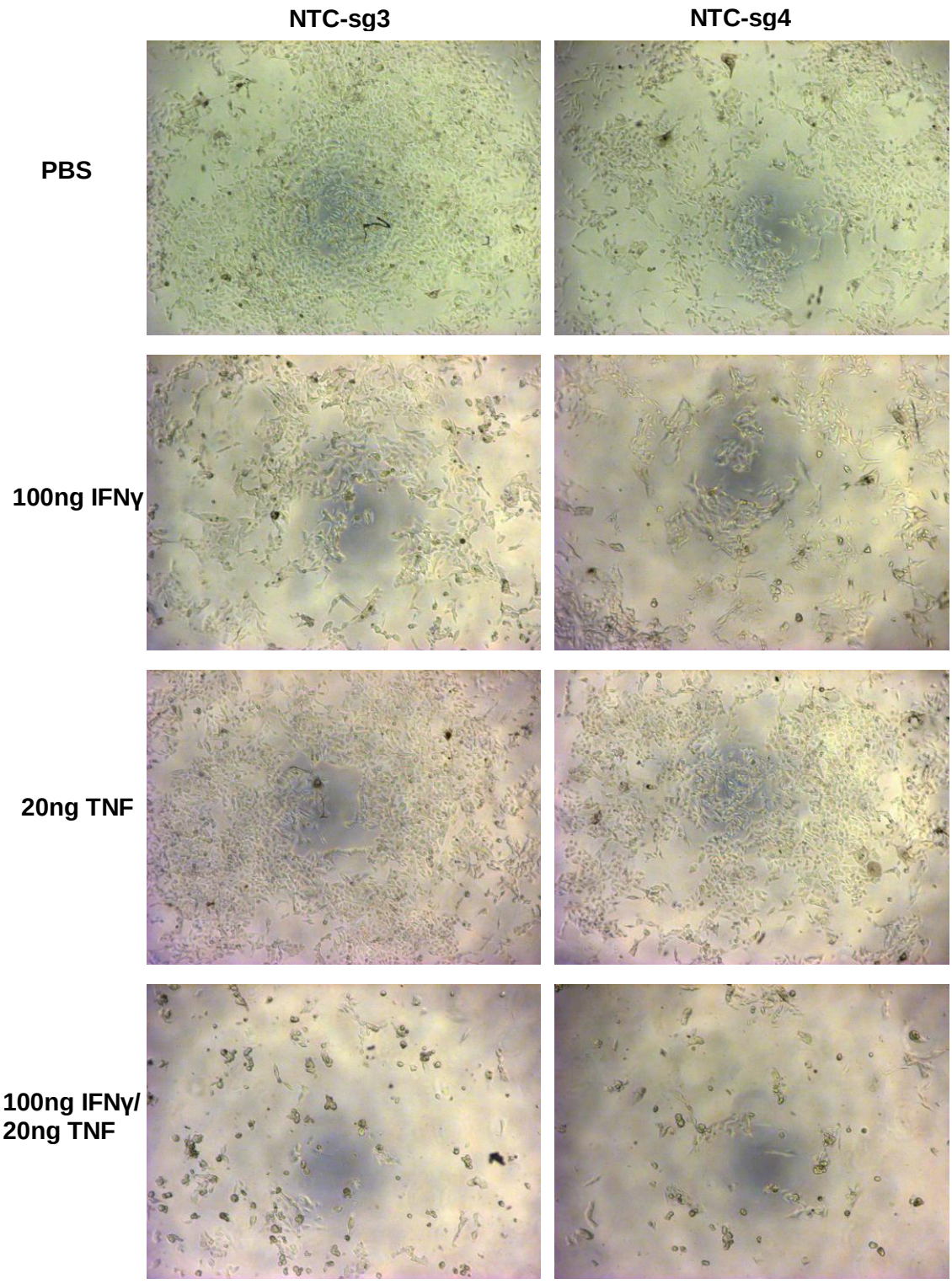


Figure 2.2.2: Continued

(C) **Control cell line with cytokines treatment (2 Days)**



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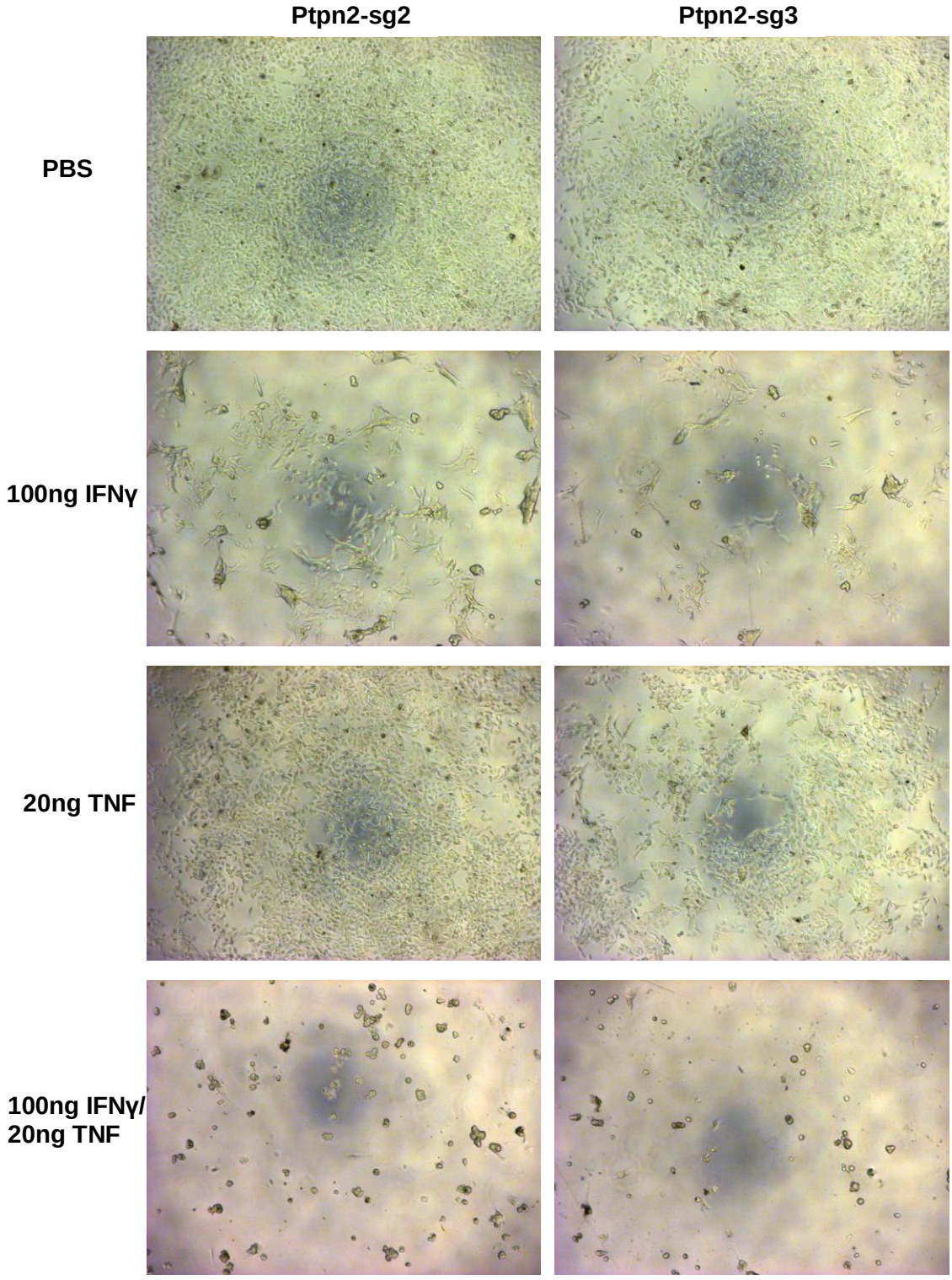

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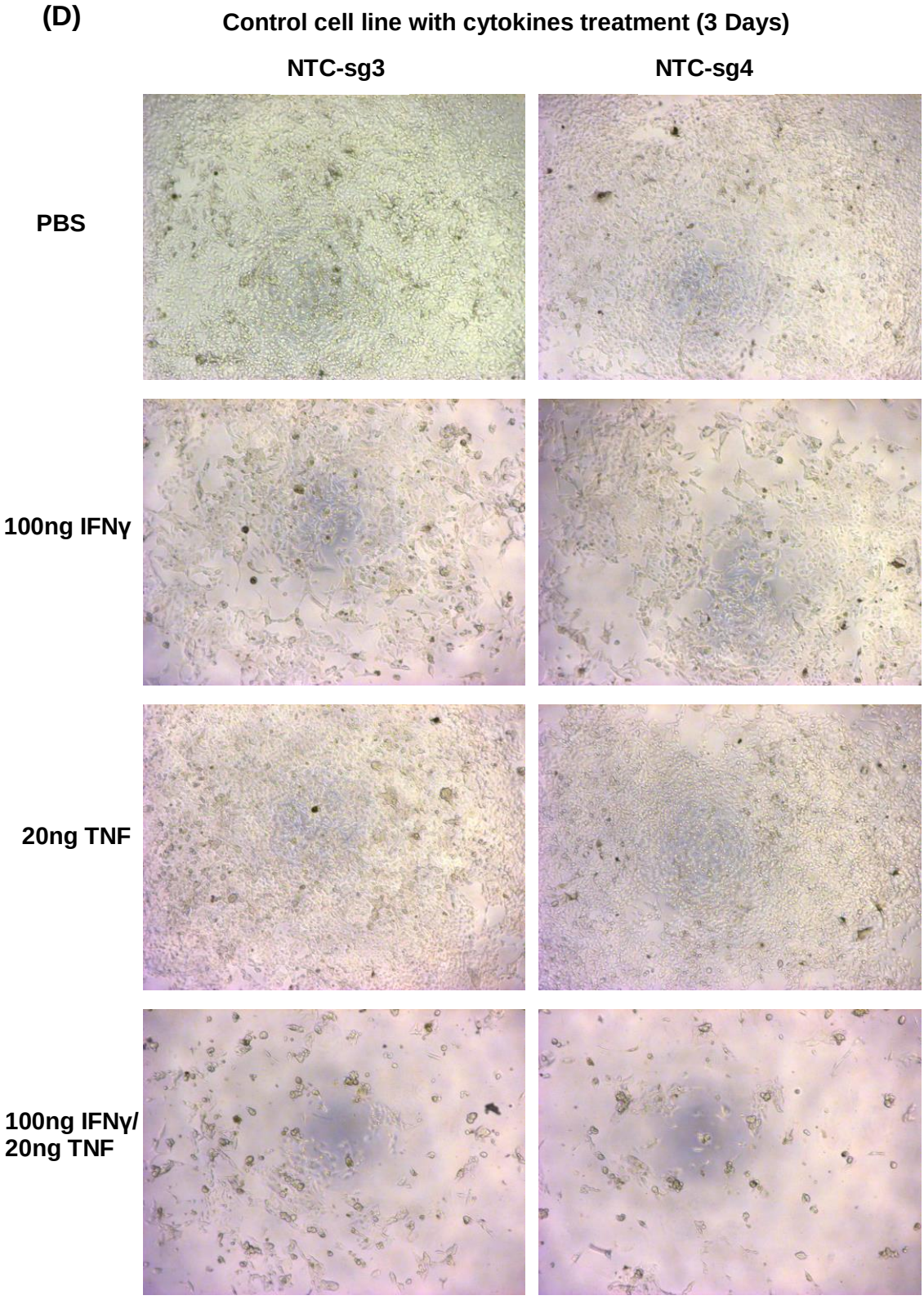

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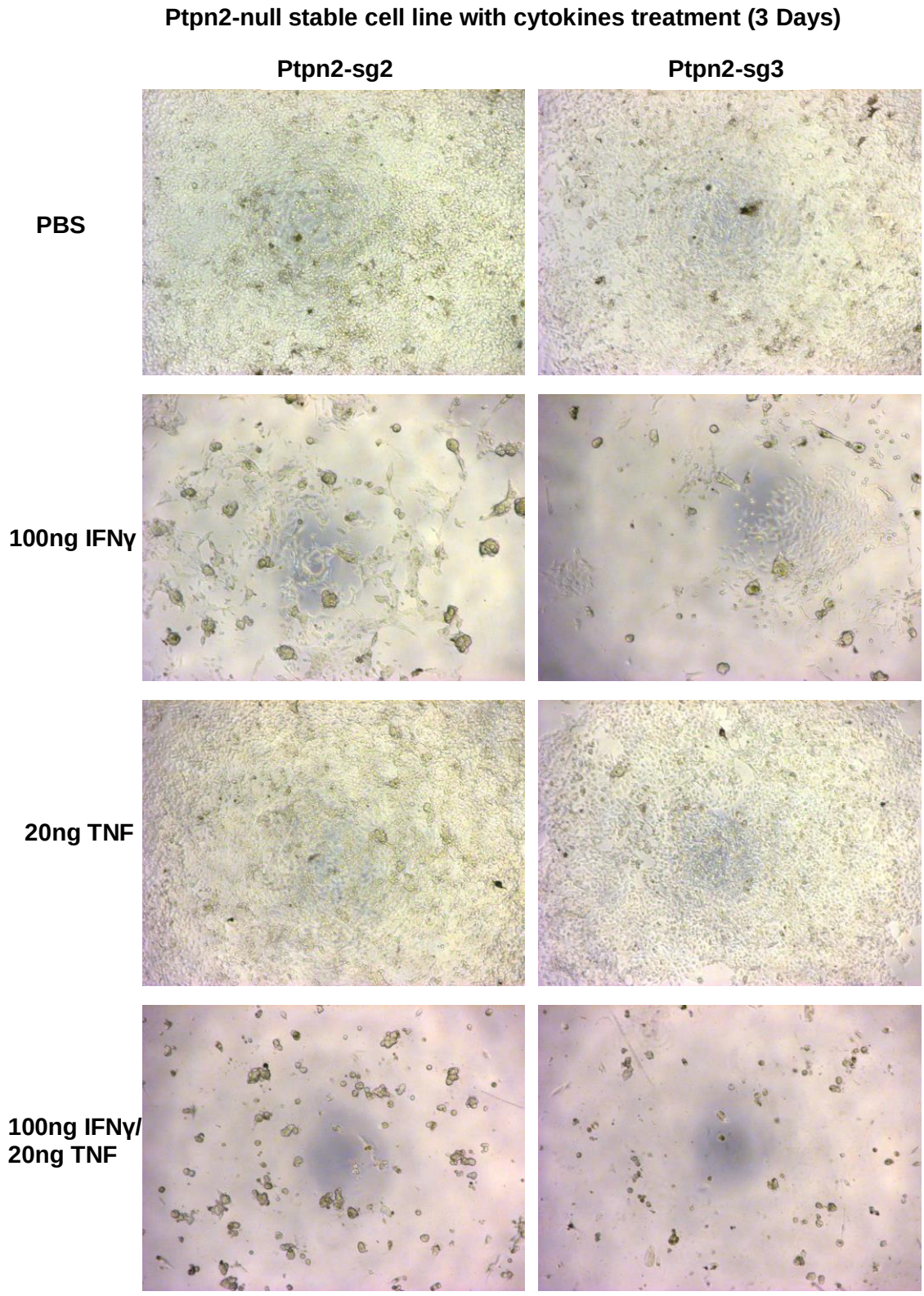
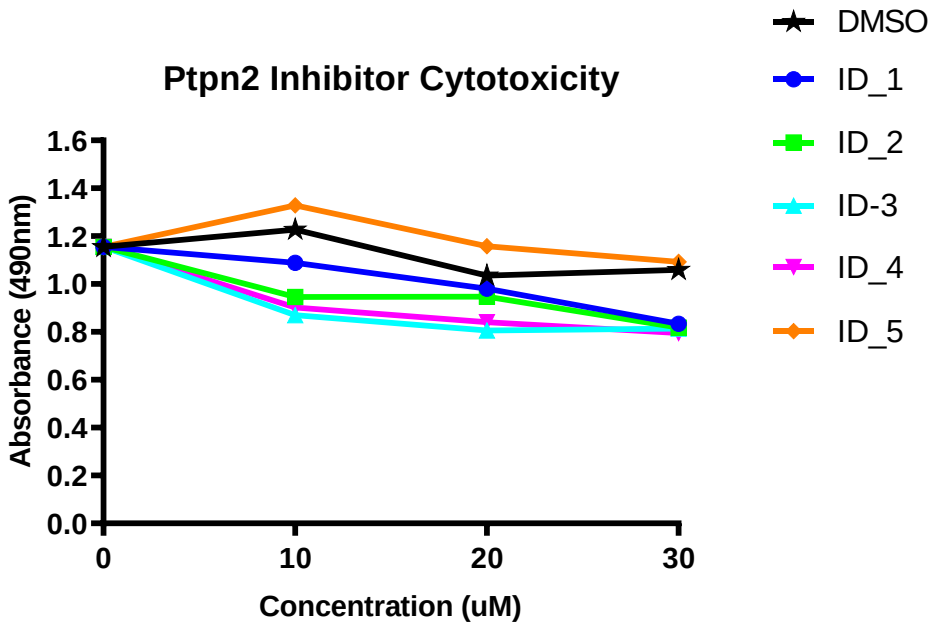


Figure 2.2.2: Continued

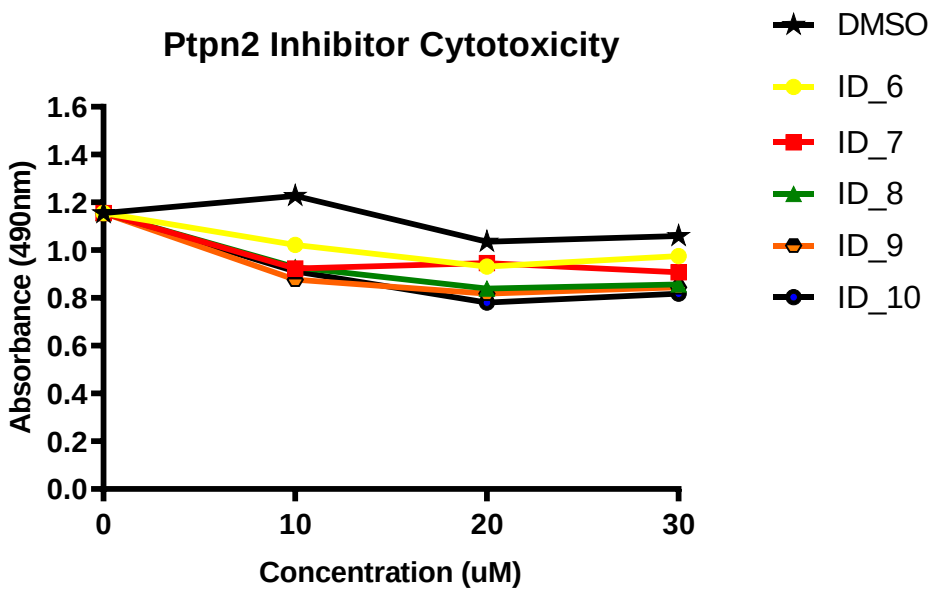
2.3 Efficacy of Ptpn2 inhibitors on murine melanoma cells

Ten compounds were evaluated for their effects on B16F10 melanoma cell line via in vitro cytotoxicity assay. The tetrazolium compound 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) containing phenazine ethosulfate (PES) assay was used to form a stable solution while recording absorbance [12]. All Ptpn2 inhibitor compounds were dissolved in DMSO. Melanoma cells were cultured in medium containing small molecule inhibitors with concentration ranging from 0 to 30 μM for 24 hours, and the cell viability was determined (Graph 2.3). Overall, the tumor cells were growing well and unaffected by cytotoxicity of DMSO at 30μM concentration.

Graph 2.3: Cell viability after drug treatment.
Melanoma cells were exposed to various concentrations of Ptpn2 inhibitors for 24 hours and cell viability was determined with MTS assay.



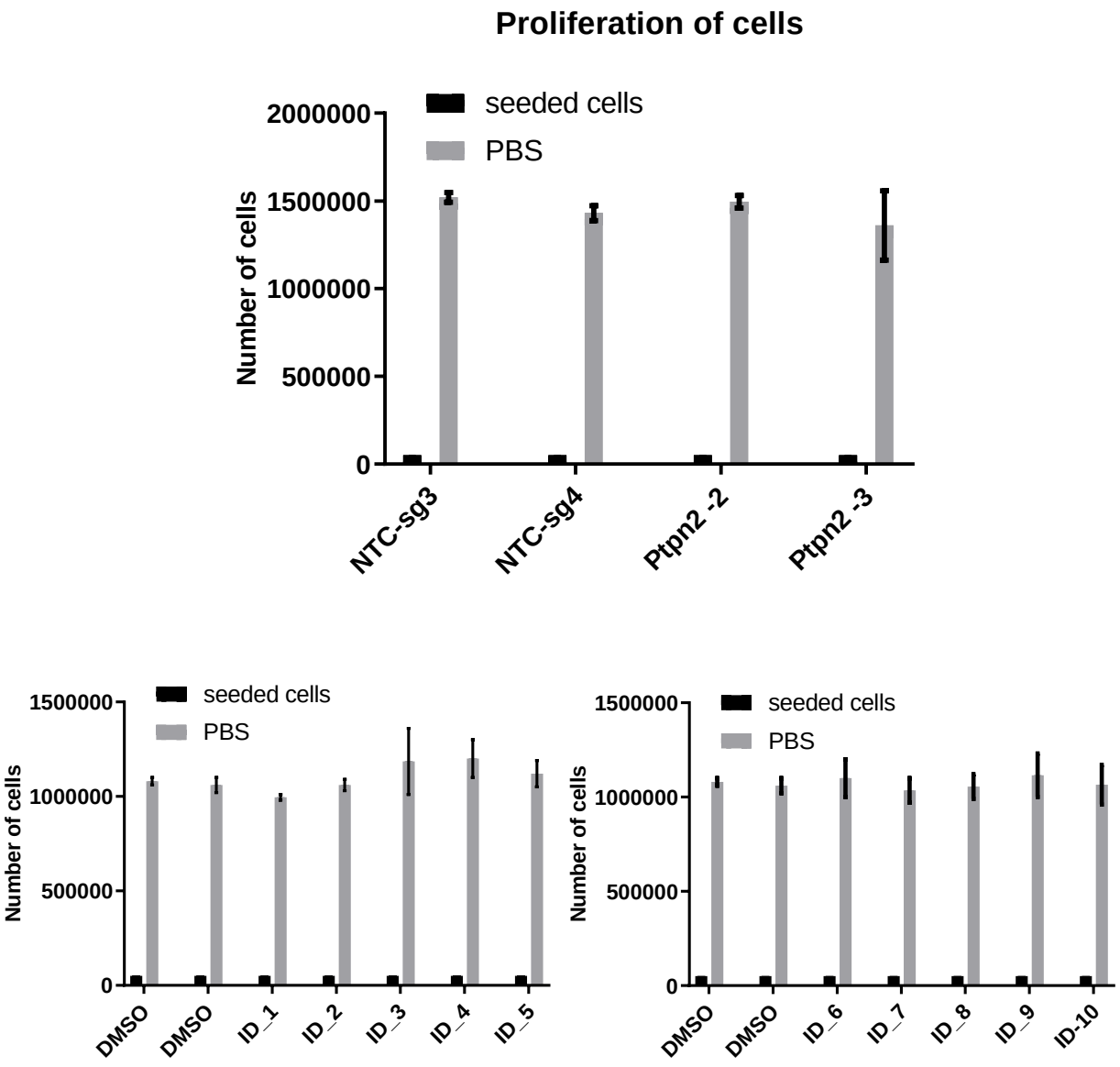
Graph 2.3: Continued



With the cytotoxicity assay, all the inhibitors had shown normal cell viability at 30µM. Hence, with the concentration determined, all the compounds were individually treated with variance conditions where cytokines and Ptpn2 small molecules were dissolved in cell culture medium and incubated together for 72 hours with: (i) 100ng/ml IFN-γ and (ii) combined 100ng/ml IFN-γ with 20ng/ml TNF-α. Then cell culture medium was replaced before extracting RNA from cells for RT-qPCR to determine gene expression levels as seen in stimulated knockout Ptpn2 cells.

Proliferation assays performed on knockout Ptpn2 cell lines had shown that the growth of cells was not affected. Hence, melanoma cells treated with Ptpn2 compounds should show similar observations. Counting live versus dead cell were determined through the cell counter with trypan blue staining. The B16F10 melanoma cells were still viable and proliferating to the inhibitory effects of the ten compounds (Graph 2.3.2). On the other hand, cells treated with cytokines and inhibitors together had not shown conclusive results.

Graph 2.3.2: Proliferation rate of control, Ptpn2-null and cells incubated with compounds for 72 hours treatment through trypan blue cell calculation with hemacytometer.

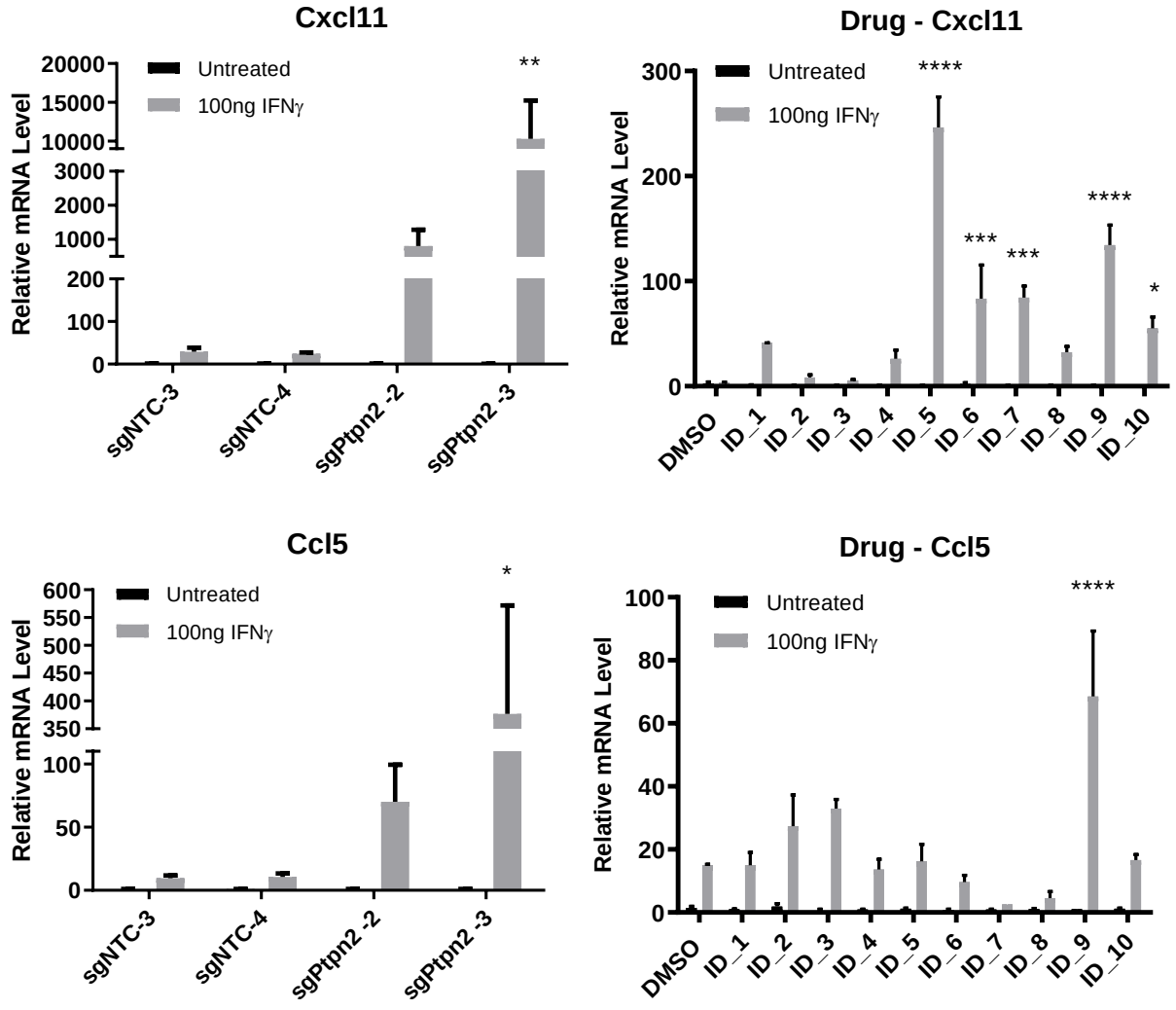


The transcriptional RNA levels in treated B16 cells with Ptpn2 inhibitors after being exposed along to IFN-γ and combined treatment are shown in Graph 2.3.2. Among the many genes that were tested in Ptpn2-null stable cell lines treated with IFN-γ and combined IFN-γ /TNF-α, T cell chemokines (Cxcl11 and Ccl5) were chosen to measure relative RNA level of each compound. IFN-γ treated cells had shown a significant increase in Cxcl11 and Ccl5 genes

expression in knockout Ptpn2 melanoma cell lines. Similarly, ID_5, ID_6, ID_7 and ID_9 had shown significant increase in cytokine gene expression.

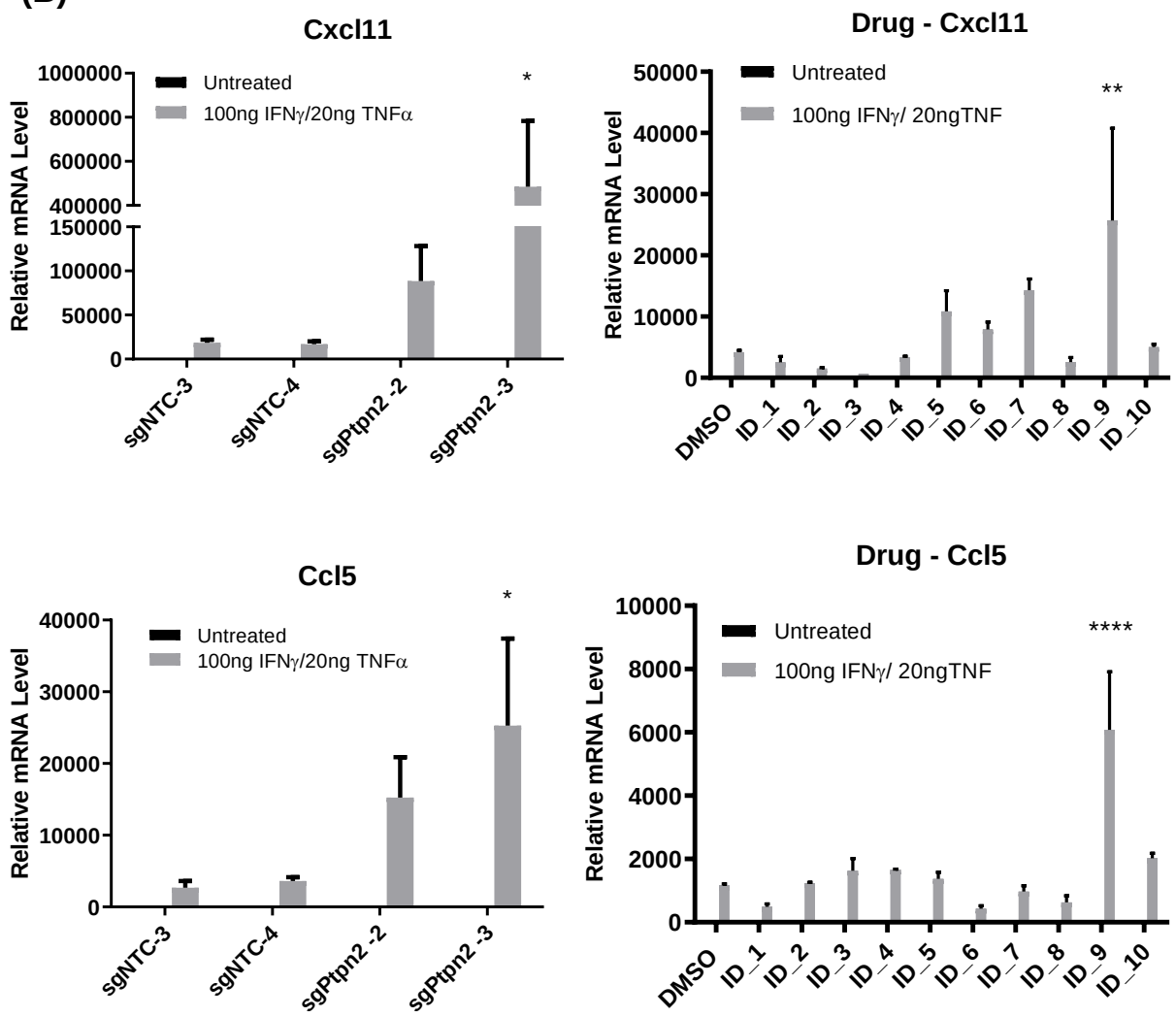
Graph 2.3.3: Quantitative transcriptional mRNA analysis of Ptpn2-null and drug treated murine cells.
After 72 hours incubation of culture medium containing (A) 100ng/ml IFN-γ and (B) combined 100ng/ml IFN-γ with 20ng/ml TNF-α were treated in B16F10 tumor cells. All assays were performed in duplicate (mean ± s.e.m., n=2 for each data point, two-way ANOVA, *P< 0.0332, **P<0.0021, ***P<0.0002, ****P<0.0001).

(A)



Graph 2.3.3: Continued

(B)



From the quantitative transcriptional mRNA analysis, compounds ID_5, ID_6, ID_7 and ID_9 had shown significant increase in cytokines expression. Hence, further experiments were performed using the selected four compounds to study phosphorylated-Stat1 and Stat1 protein through western blot. Wild type B16 cells were treated with four compounds for 12 hours before changing the media containing 100ng/ml of IFN-γ for another 24hours. As shown in Figure 2.3 and 2.3.2, untreated cells did not express phosphorylated-Stat1 but only Stat1 protein. In comparison to Ptpn2-null cells where phosphorylated-Stat1 was highly upregulated, B16 cells treated with ID_5, ID_7, and ID_9 compounds had expressed phosphorylated-Stat1 stronger

than wildtype B16 cells treated in same condition which showed faint bands. High expression of unphosphorylated-Stat1 in treated cells with IFN-γ was also similarly seen in quantitative RT-PCR in Graph 2.2 where control B16 cells exposed to cytokine had upregulated Stat1 genes. Thus, these results demonstrated that inhibition of Ptpn2 ID_5, ID_7, and ID_9 compounds with exposure to IFN- γ alone is able to sensitize tumor cells, similarly to Ptpn2-null stable cell lines.

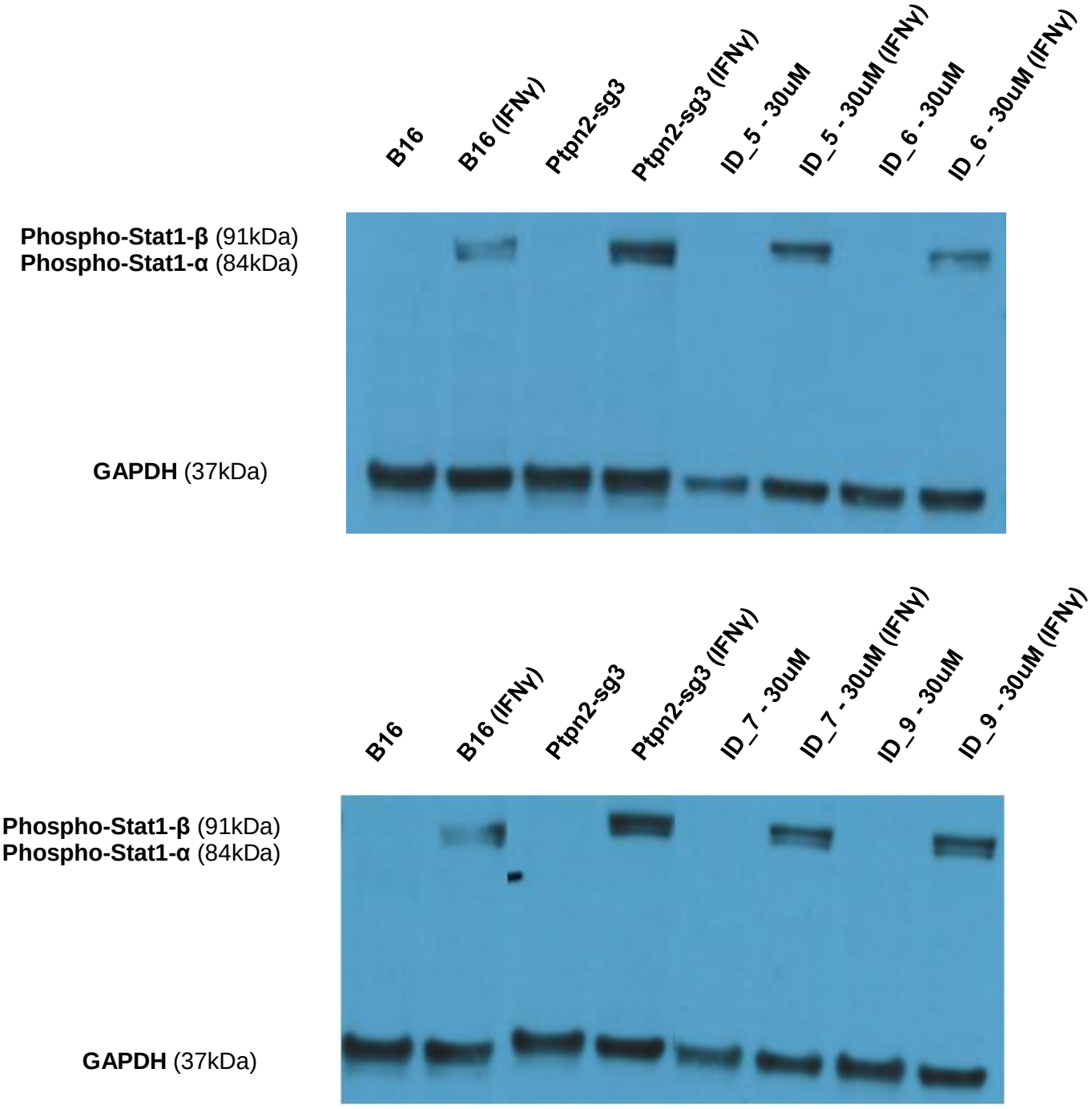


Figure 2.3: Western blot of phosphorylated-Stat1 after 24 hours of IFN-γ treatment to control, Ptpn2-null and treated B16 cell with Ptpn2 inhibitors ID_5, ID_6, ID_7, and ID_9.

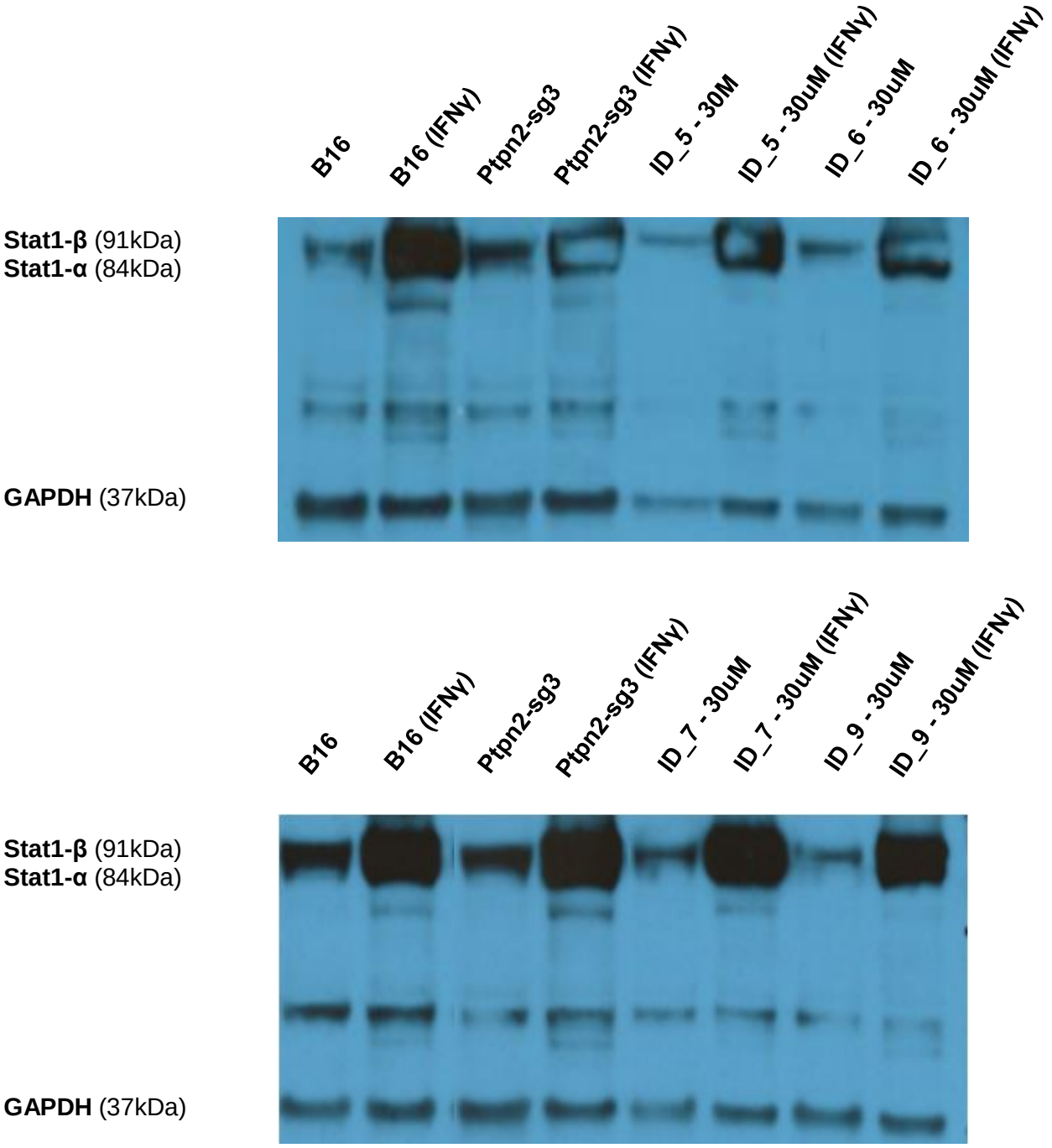


Figure 2.3.2: Stat1 after 24 hours of IFN-γ treatment to control, Ptpn2-null and treated B16 cell with Ptpn2 inhibitors ID_5, ID_6, ID_7, and ID_9 shown through western blot.

Chapter 3: Discussion and Future Perspective

Ptpn2 is a protein tyrosine phosphatase that negatively regulates interferon gamma (IFN-γ) signaling. It dephosphorylates Stat1 and Jak1 which are involved in type 1 and type 2 of the interferon gamma pathway. Hence, the deletion of Ptpn2 has marked the increase response of tumors to IFN-γ which invoked growth arrest. This further suggests that therapeutic inhibition of Ptpn2 effects could sensitizes tumors to immunotherapy [2].

The use of virtual high throughput screening (vHTS) and rational structural design of small molecules had led to the identification of Ptpn2 inhibitors ID_5, ID_7 and ID_9. These compounds were tested along with positive control Ptpn2-null murine melanoma cell line. These leading Ptpn2 inhibitors had shown significant increase in phosphorylation of Stat1 protein as seen in knockout Ptpn2 cell line after being treated with IFN- γ. These results may potentiate the future treatment with immunotherapy to patients that induce an IFN-γ response.

Additional work needs to be completed for *in vivo* studies such as quantitative RT-PCR with quantitative immunoblotting, and optimizing drug dosage *in vitro* to further determine whether compounds are effective against tumor growth. Moreover, further improvement of compounds' structures to enhance aquoeus solubility would be an approach for future works. Additional *in vivo* experiments will be performed through intratumoral and systemic injections into mice with melanoma tumors.

Current advancement in immunotherapy to treat cancer patients has shown many great response rates. However, the subset of patients with negative response to treatment has also led to growing a comradery among cancer researchers to further pursue research to improve response rate in treatments. Other than focusing on functional genomic approaches by using genomic editing, inhibition of Ptpn2 encoding protein tyrosine phosphatase through proper structural design of small molecules may provide therapeutic window to selective patients. Cancer patients undergoing PD-1 or CTLA4 immunotherapy may be able to incorporate these Ptpn2 compounds for better outcomes.

Chapter 4: Material and Methods

4.1 Cell culture

Mouse melanoma cell line (B16F10) were grown and maintained in DMEM (Gibco) supplemented with 15% of heat inactivated FBS, 2mM of L-glutamine and 100U/ml Penicillin Streptomycin. All cell lines were cultured in 37°C incubator with humidified atmosphere of 5% carbon dioxide. Trypsin-EDTA (1X) was used to detach cells from culturing plate surface.

4.2 CRISPR-knockout tumor cell lines

4.2.1 Lentivirus Vector Generation

Following the Zhang lab's protocol to generate lentiviral vector [10], synthesized oligonucleotides with target guide sequence were digested, annealed and cloned into the lentiCRISPRv2. Plasmids were transformed into Stbl3 bacteria. Successfully transformed bacteria able to proliferate on agar plates and LB broth containing ampicillin (1:1000 dilution) for 12 hour. Plasmids were collected from bacteria using Qiaprep Spin Miniprep Kit.

Table 4.2.1: CRISPR target guide sequence to generate stable cell lines.

CRISPR sgRNA sequences	
NTC – sg1	GCGAGGTATTCGGCTCCGCG
NTC – sg2	GCTTTCACGGAGGTTCGACG
NTC – sg3	ATGTTGCAGTTCGGCTCGAT
NTC – sg4	ACGTGTAAGGCGAACGCCTT
Ptpn2 – sg1	CCATGACTATCCTCATAGAG
Ptpn2 – sg2	TCATTCACAGAACAGAGTGA
Ptpn2 – sg3	ATGTGCACAGTACTGGCCAA

4.2.2 Transfection

Lentiviral production was performed in a 6-well culturing plate using HEK293T cells. Transfection reagents were separated into Tube A and Tube B where Tube A contained Lipofectamine 2000 Reagent (Invitrogen) and Optimem. Tube B had both packaging plasmids (PsPAX and PMD2.G) and lentivector with ratio of 3:1:4 respectively. Both mixtures were vortex

until homogenous. Immediately, both tubes were combined and incubated under room temperature for 15-20 minutes. Then, the transfection complex was transfered into 10cm culturing dish containing HEK293T cells to incubate for 24 hours before collecting the virus.

4.2.3 Transduction

B16F10 cells were seeded into a 6-well culturing plate where the day of transduction achieves about 70% confluency. In each wells, lentivirus and Polybrene (Millipore-Sigma) were added. Spin-transfection transduction was performed for 1.5 hours under 2000rpm. The following day (after 24 hours incubation), the media in each well was replaced with fresh media containing 1ug/ml – 1.5ug/ml Puromycin (Alfa Aesar) for positive selection.

4.3 *In vitro* cytokine stimulation

B16 melanoma cells were seeded a day before in a 12-well plate stimulation with cytokines where 50% confluency was achieved the following day. The two cytokines being used were Recombinant Mouse IFN-gamma (Animal-Free) Protein (BioLegend) and TNF-alpha (Carrier-Free) Protein (Biolgend). Ptpn2 small -molecules were dissolved in 100% DMSO.

4.4 Small molecule inhibitor treatment

Murine melanoma B16F10 cells were seeded a day before treatment in a 12-wells plate where about 50% confluency was achieved the following day. This was followed by adding Ptpn2 inhibitor compounds in cell medium containing 30μM each drug or dimethylsulfoxide (DMSO) as a control. After 72 hours incubation of small compound inhibitor with cytokines treatments, cellular RNA were extracted.

4.5 RNA extraction and real-time PCR

Total RNA was isolated from cells using TRIzol Reagent (Invitrogen) and subjected (1000ng RNA) to reverse transcription with iScript Reverse Transcription Supermix for complementary DNA (cDNA) generation following manufacturer's instructions. Quantitative RT-PCR was performed via iTaq Universal SYBR Green Supermix (BioRad) using Roche's Light

Cycler 480 II. Normalization and fold changes for each genes' expression were quantified using the comparative Ct method with GAPDH as an internal control.

Table 4.5: The primers used for RT-PCR.

Primers used for RT-PCR			
Gene name	Upper primer (5'-3')	Lower primer (5'-3')	Species
Cxcl 11	GGCTGCGACAAAGTTGAAGT	CGAGCTTGCTTGGATCTGGG	Mouse
Ccl 5	GTTCCATCTCGCCATTCATGC	TAAGCAAACACAACGCAGCTC	Mouse
Tap 1	TTCACCCGCAACATATGGCT	ATGTGATGGAACCTGCTGGG	Mouse
Stat 1	GGCCTCTCATTGTCACCGAA	TACCACAGGATAGACGCCCA	Mouse
Stat 2	GTCGTCTTCAGACCCCCATC	GCCAACCAGTCCTTTGGAGA	Mouse
Stat 3	GGAACAGATGCTCACAGCCC	AGTCAGTGTCTTCTGCACGTA	Mouse
P21	TGGACAGTGAGCAGTTGCG	CGTCTCCGTGACGAAGTCAA	Mouse
Casp 8	TAGAAGGCTACCAAAGCGCA	CCCTTGTCACCGTGGGATAG	Mouse
Pd-l1	TGGTGGAGTATGGCAGCAAC	CCCAGTACACCACTAACGCA	Mouse
Irf 1	GGAGATGTTAGCCCGGACAC	AGGTAGCCCTGAGTGGTGTA	Mouse
Irf 9	CCCGAGAGAGGTCGTATGGA	TGGTTCCGTGGTTGGTTAGG	Mouse
Ptpn2	GGCCAACGGATGACAGAGAA	GGTCAGGGGTCAAACAACCA	Mouse
Gapdh	AAGGTCATCCCAGAGCTGAA	CTGCTTCACCACCTTCTTGA	Mouse

4.6 Proliferation assay for Cytokines treatment

Cellular proliferation after cytokines treatment with IFN-gamma and TNF-alpha were calculated using hemocytometer. Dead and live cells were differentiated with Trypan Blue 0.4% (Lonza). Cell images were taken using Leica fluorescence microscope (DMI 3000B) (Leica Microsystem, Wetzlar, Germany).

4.7 MTS assay

A day before drug stimulation, B16F10 cells were seeded in a 96-well plate. The cellular viability for cells being treated with DMSO and Ptpn2-inhibitor were performed using CellTiter96 Aqueous One Solution Reagent (Promega). 20µl of MTS reagent into each well for 1-4 hours incubation at 37°C and measured for absorbance at 490nm using a Synergy 2 BioTek plate reader.

4.8 Western bloting and antibodies

Before treating B16 melanoma cells with Ptpn2 inhibitor for 12 hours in a 6-wells plate, murine cells were seeded a day before to ensure that about 50% confluency. The cell medium was replaced with medium containing 100ng/ml IFNγ for another 24 hours. B16 cells were lysed in lysis buffer (60mM Tris HCL, 2%SDS and 10% glycerol) containing Benzoate Nuclease (Sigma Aldrich), DTT, PMSF and Protease Inhibitor Cocktail 3 Mammalian (RPI). Lysate were incubated in ice for 15 mins and then centrifuged at 13,000rpm for 20 mins at 4°C. Protein supernatant concentration were quantified using DC Protein Assay (BioRad) with Synergy 2 BioTek plate reader measuring at 750nm. 50-80µg of proteins were denatured at 95°C for 8 mins before loading into NuPAGE Novex 4-12% Bis-Tris Gel 1.5mm (Invitrogen). Ladder used to estimate protein size was SeeBlue Plus2 Pre-stained Protein Standard (ThermoFisher). PVDF membrane (BioRad) were incubated overnight with 1% BSA before blotting with antibodies and washed with 1X PBST containing Tween 20 (BioRad). Membranes were developed by Pierce ECL Western Blotting Substrate (ThermoFisher).

For western blotting, the antibodies used are Phospho-Stat1 (Tyr701) (D4A7) Rabbit mAb Antibody (Cell Signaling 1:1300), Stat1 Rabbit mAb Antibody (Cell Signaling 1:1300), Anti-rabbit IgG, HRP-linked Antibody (Cell Signaling 1:1000), and GAPDH (14C10) Rabbit mAb (HRP Conjugate) Antibody (Cell signaling 1:1000).

4.9 Statistical Analysis

GraphPad Prism v.8.0a program was used to perform all statistical test and graphing of data. Not depicted otherwise, P-values were calculated using two-way ANOVA (or mixed model) for multiple comparison for Ptpn2 inhibitors. On the other hand, P-values were calculated for knockout Ptpn2 stable melanoma cell lines using one-way ANOVA (and nonparametric/mixed) and Bonferroni-Sidak method.

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